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# AUGMENTATION OF CASTRATE URINARY GONADOTROPIN BY A NON-SPECIFIC URINARY COMPONENT[1]

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AUGMENTATION of the ovary stimulating effect of anterior pituitary gland extracts by the addition of non-specific substances has been frequently noted. Among the substances found to have such an effect are tannic acid (Fevold, Hisaw, Hellbaum and Hertz, 1933), zinc sulphate (Maxwell, 1934; Saunders and Cole, 1936), the formed elements of cattle blood (Casida, 1936), casein and egg albumin (Saunders and Cole, 1936), and extracts of milk, egg, liver, thyroid, and lemon (Hellbaum, 1936). On the other hand, no such effect of non-specific substances on the gonadotropic extract from castrate urine has been demonstrable. The results reported below may explain this fact.

In the course of experiments with the gonadotropic substance excreted by ovariectomized women, the untreated urine was thoroughly dialyzed against water, using a cellophane "suasage casing" membrane. Assays of the urine before and after dialysis showed a considerable loss of potency to have occurred during this process. Such loss of activity during dialysis has been noted by others (Levin, 1941; Varney and Koch, 1942), but no explanation for it has been reported. Because of the magnitude of the decrease in activity it seemed advisable to investigate its cause. The results of this investigation are reported in this paper.

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*Experimental.* The urine used in these experiments was obtained from surgically castrated subjects (5 women; 1 man). It was collected over chloroform and refrigerated until use. Assays were done by the mouse uterine weight method of Levin and Tyndale (1937) on aqueous extracts prepared from alcohol precipitates. Aliquots of the untreated urine and of the dialysed urine were precipitated by addition of 5 volumes of 95 per cent ethanol as soon as dialysis was terminated. The precipitated material was collected at the centrifuge, washed with ether, freed of ether in vacuo and retained as the dry powder. Just prior to assay the powder was dissolved in an appropriate amount of water.

It was found that the duration of dialysis had no effect on activity. Portions of a specimen, subjected to dialysis for 13, 18, 24, 30 and 36 hours respectively, all showed the same loss of activity. Neither the size of the membrane nor the brand (Cenco and Visking brands were used) influenced the amount of activity lost. The temperature of the water against which dialysis was carried out did have some effect. A greater loss of activity occurred at room temperature than at temperatures of 10° C. or less. This did not, however, account for the total loss observed.

The possibility presented itself that some dialysable substance normally present in urine might cause an augmentation of the gonadotropic effect and that the removal of such a substance might result in an apparent loss of gonadotropic activity.

To test this possibility, known volumes of the untreated urine were evaporated to dryness over a steam bath and the dry residue was heated for several hours to complete the destruction of any heat-labile gonadotropic substances. Repeated assays were done on such residues to determine if any activity remained. Amounts equivalent to 20 cc. of the original urine were entirely inactive. Attempts to assay the heated residue at higher levels were unsuccessful because of toxicity. Despite the apparent inactivity of this residue, it was found that the addition of an equivalent amount of it to the dialysed urine completely restored activity to the original level where dialysis had been done at low temperatures. The incomplete restoration of activity where dialysis occurred at room temperature probably results from an actual loss of gonadotropic activity, caused by the prolonged exposure to the higher temperature. The results are given in table 1.

It seemed possible that addition of the urinary residue increased activity merely by the mechanical effect of causing more complete alcohol precipitation of the gonadotropin from its salt-free solution. To test this, the residue was added to one dialyzed specimen prior to alcohol precipitation while an identical dialyzed specimen was precipitated alone and its aqueous extract was mixed with an extract of the residue. The data for D26C and D26D and from D27C1 and D27D1 indicate that such difference in treatment causes no signifi-

cant variation in results. The data obtained (D27C and D27D) tend to confirm the view that a true augmentation is involved for addition of as little as $\frac{1}{3}$ the equivalent amount of residue effectively restored activity, while $\frac{1}{4}$ the equivalent amount of residue produced no appreciable augmentation.

An attempt was made to determine what substance in the urinary residue was responsible for the augmentation. A solution was pre-

TABLE 1

| Preparation | Treatment | | | Minimal Active Dose | Yield |
|---|---|---|---|---|---|
| | Dialysis | | Add'n. Residue | | |
| | *Hrs.* | *°C.* | | *ml. urine* | % |
| D27A | no dialysis | | | 4 | |
| D27B2 | 21 | 21 | none | 8 | 50 |
| D27C2 | 21 | 21 | equiv. am't. | 5 | 80 |
| D25A | no dialysis | | | 3.5 | |
| D25B | 26 | 4–8 | none | 7 | 50 |
| D25C | 26 | 4–8 | equiv. am't. | 3.5 | 100 |
| D29A | no dialysis | | | 12 | |
| D29B | 29 | 5–10 | none | >24 | <50 |
| D29C | 29 | 5–10 | equiv. am't. | 12 | 100 |
| D33A | no dialysis | | | 5 | |
| D33B | 30 | 2–3 | none | 8 | 63 |
| D33C | 30 | 2–3 | equiv. am't. | 5 | 100 |
| D26A | no dialysis | | | 3.5 | |
| D26B | 28 | 22 | none | 6 | 58 |
| D26C | 28 | 22 | equiv. am't. before alc. pptn. | 4 | 87 |
| D26D | 28 | 22 | equiv. am't. to final extract | 4.5 | 78 |
| D27A | no dialysis | | | 4 | |
| D27B1 | 22 | 21 | none | >7 | <57 |
| D27C1 | 22 | 21 | equiv. am't. before alc. pptn. | 5 | 80 |
| D27D1 | 22 | 21 | equiv. am't. to final extract | 5 | 80 |
| D27A | no dialysis | | | 4 | |
| D27B | 24 | 21 | none | 9 | 44 |
| D27C | 24 | 21 | $\frac{1}{2}$ equiv. am't. | 4.5 | 89 |
| D27D | 24 | 21 | $\frac{1}{4}$ equiv. am't. | 7 | 57 |
| D31A | no dialysis | | | 15 | |
| D31B | 52 | 2–3 | none | 15 | 100 |
| D31C | 52 | 2–3 | equiv. am't. | 15 | 100 |

pared, containing in their physiological proportions the following dialysable, heat stable components of normal urine: Na, K, Ca, Mg, Cl, $SO_4$, $PO_4$ and uric acid. Addition of this mixture to dialysed specimens of castrate urine was not effective in restoring or increasing the activity. Some other substance must be involved.

It is interesting in this connection to note the results for D31A, D31B and D31C. Only one specimen, taken after the ingestion of creatine, could be obtained from this male castrate. No change in activity, despite prolonged dialysis, occurred. Unfortunately it was

not possible to determine if his sex, or the ingestion of creatine had any bearing on the divergent behavior of this specimen. However, since the other results were obtained on the urines of five different castrate women, it seems reasonable to assume that the augmenting substance is a usual component of such urines.

*Conclusion.* The activity of the gonadotropin in the urine of castrate women is augmented by a dialyzable substance present in the same urine. In view of this, findings based on assays of undialyzed extracts of alcohol precipitates of such urine do not give the true value of gonadotropic activity, and their revision is indicated.

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# EFFECTS OF ADRENOCORTICOTROPIC HORMONE (ACTH) ON THE OSSEOUS SYSTEM IN NORMAL RATS[1]

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In 1937, Moon reported that the body weight gain of young castrate rats was retarded by the injection of anterior pituitary extracts rich in the adrenocorticotropic factor. There is now conclusive evidence from the injection of the pure hormone (Evans, Li and Simpson, 1943) that the stunting produced by the cruder extracts was due to this hormone. The present paper reports the characteristic effects of this hormone on chondrogenesis and osteogenesis in the epiphyseal region of the tibia.

## ADRENOCORTICOTROPIC HORMONE IN NORMAL YOUNG RATS

Twelve male rats 26 days of age were injected intraperitoneally with pure adrenocorticotropic hormone (ACTH) for thirty days thrice daily, the total daily dose being 1 mg. A corresponding number of rats was used as controls. In half the experimental and half the control rats, food consumption was measured.

As will be seen in Table 1, the injected rats gained only half as much in body weight as the controls during the experimental period (80 gm. compared with 154 gm.). This occurred in spite of the fact that they consumed the same amount of food as their controls (12.0 gm. daily, controls 12.8 gm., a difference which is within the accuracy of measurement). The total body length of the injected rats (nose tip to tail tip) was also considerably less than that of their controls at autopsy.

The growth retardation as expressed in the appearance of the proximal epiphyseal cartilage of the tibias of the animals treated with ACTH is seen in Figures 1 to 4. The control animals showed an average width of the epiphyseal cartilage of 409 micra while that of the

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experimental animals was 249 micra. Osteogenesis in the controls was very active, but greatly retarded in the ACTH treated animals. This is best shown in Figures 3 and 4 which are at higher magnification. The more active osteogenesis in the controls was indicated by the larger numbers of osteoblasts. Under ACTH treatment the zone of osteogenesis did not extend into the shaft so far as in the normal rat; the zone of erosion was more irregular, and contained fewer of the large, vacuolated cartilage cells. The irregular bony trabeculae of the injected animal is to be compared with the slender, straight trabeculae of the normal rat. The marrow sinusoids were wider than those in the control. The capillaries in the zone of erosion were also unusually distended.

A comparison with hypophysectomized animals (which were sacrificed at a postoperative period of the same duration as the period of injection of the normal rats with ACTH) indicates that in hypophysectomized rats osteogenesis was completely arrested as seen in Figure 5. The width of the epiphyseal cartilage was more reduced than in the ACTH treated animals and osteogenesis had ceased, the cartilage having been "sealed off" from the marrow cavity, whereas in the normal rat injected with ACTH, osteogenesis continued at a restricted rate.

### COMPARISON OF THE EFFECTS OF FOOD RESTRICTION WITH THOSE OF ACTH INJECTIONS INTO NORMAL YOUNG RATS

As the rats dwarfed by ACTH ate substantially the same amount of food as their controls, limitation of food could not have been the cause of the differences observed between injected and controls. The animals might have been starved, however, in the sense that oversupply of ACTH resulted in wasteful metabolic changes. For this reason comparisons of growth and bony structures were made on rats dwarfed by food restriction. Table 2 shows the effect on body growth of food restriction for 30 days in 26 day old male rats. Only 8.9 gm. of food daily were allowed, whereas controls consumed 12.8 gm. daily. By comparison with Table 1, it will be seen that the final body weight

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PLATE I. Proximal epiphyseal regions of the left tibias of normal 56 day old rats; hematoxylin and eosin.

FIG. 1. Normal untreated control. Very active endochondral bone formation is shown. ×50 (Spec. 7399, Pl. 7407).

FIG. 2. Normal animal, receiving injections of 1 mg. ACTH daily for 30 days: width of epiphyseal cartilage is considerably smaller than in control. Osteogenesis is greatly retarded. Bony trabeculae are crowded and irregularly arranged. ×50 (Spec. 7394, Pl. 7422).

FIG. 3, 4. Magnifications of erosion zones and adjacent regions of endochondral bone formation of Figures 1 and 2. Osteoblastic and osteoclastic activities are very marked in the normal, while they are diminished in the ACTH treated animal. ×150 (Pl. 7930, 7931).

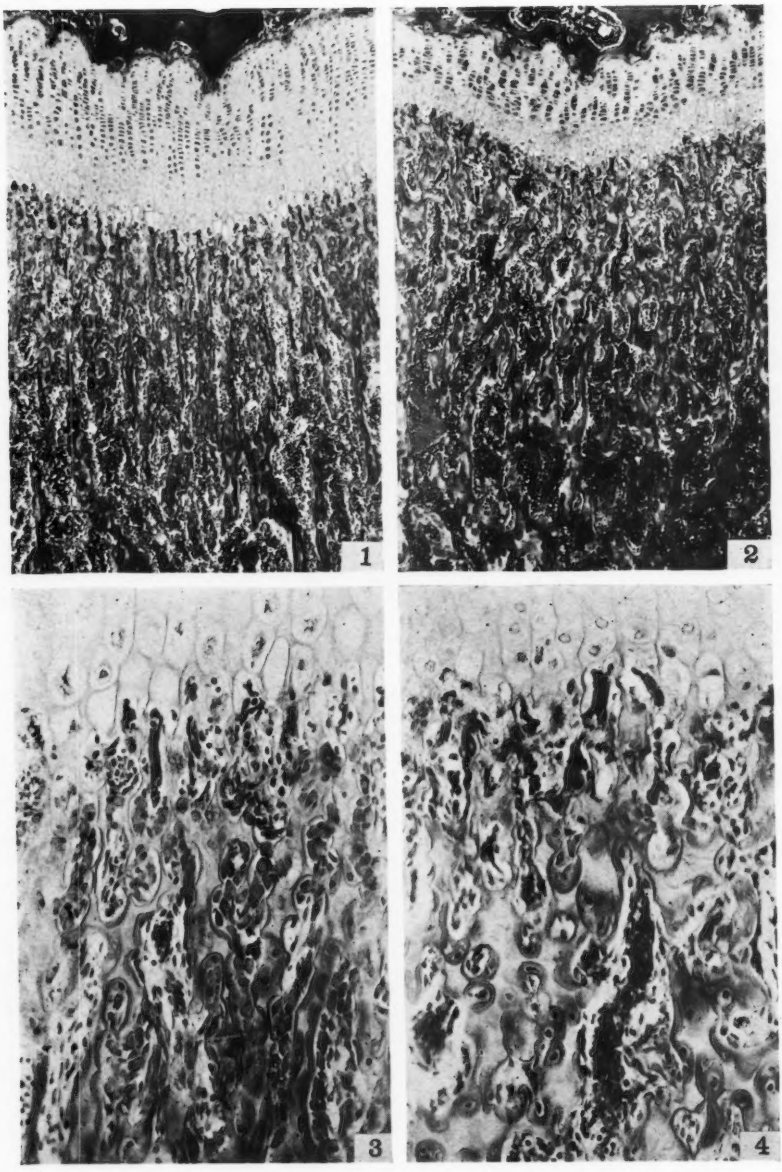
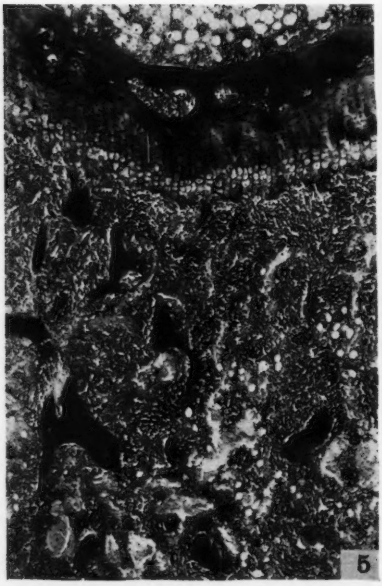



PLATE I, Figs. 1–4. See opposite page for description.



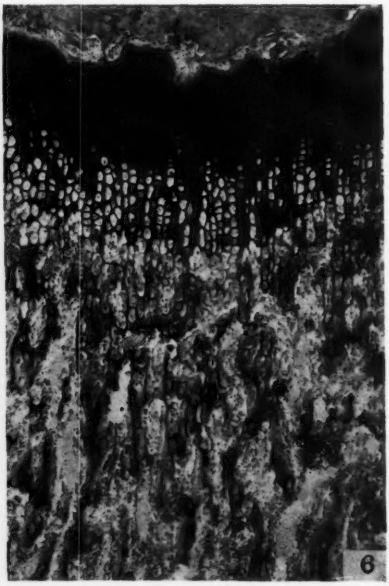



PLATE II. Proximal epiphyseal cartilages of left tibias of rats, 56 days of age at autopsy, central sagittal sections; hematoxylin and eosin, 8 micra, ×50.

FIG. 5. Hypophysectomized animal. Osteogenesis has ceased; epiphyseal cartilage is being "sealed off" by osseous tissue against the marrow cavity. Only a few fragments of bony trabeculae are seen. (Spec. 6905, Pl. 7978).

FIG. 6. Normal animal, food ad libitum (measured). (Spec. 7550, Pl. 7927).

FIG. 7. Normal animal, receiving approximately two thirds the diet of the control (Fig. 6). Cartilage width is reduced (Spec. 7557, Pl. 7918).

and body weight gain during the experimental period were somewhat larger than in the ACTH injected group, the rats on restricted food being retarded behind their controls by 63 gm., whereas ACTH caused a retardation of 74 gm. Retardation in growth in body length was more nearly comparable, the rats on restricted food being 3.5 cm. shorter from the nose to tail-tip than their controls, the ACTH treated rats being 3.7 cm. shorter than their controls.

The proximal epiphyseal cartilage as seen in Table 2 and Figures 6 and 7 was somewhat narrowed, being 93 micra narrower than that

TABLE 1. GROWTH RETARDATION FROM INJECTION OF ADRENOCORTICOTROPIC HORMONE* FOR 30 DAYS IN *Normal* 26 DAY OLD MALE RATS

| Treatment | No. of Rats | Final Body Weight | Body Weight Gain in 30 Days | Final Length Nose to Tail-tip | | Width‡ of Proximal Epiphyseal Cartilage of Tibia | | Av.§ Daily Food Intake |
|---|---|---|---|---|---|---|---|---|
| ACTH | 12 | *gm.* 145 (110–175) | *gm.* 80 (51–105) | *cm.*† 32.2 (30.6–33.8) | *SE* ±0.27 | *micra* 249 (211–322) | *SE* ±7.9 | *gm.* 12.0 (10.8–13.8) |
| Control | 12 | 219 (176–252) | 154 (120–183) | 35.9 (33.5–38.9) | ±0.46 | 409 (333–504) | ±11.7 | 12.8 (11.8–13.7) |

* Pure sheep pituitary adrenocorticotropic preparations, L1234DP and L1249F, daily dose 1 mg. intraperitoneally, divided in 3 injections during 8 hour period.
† Measurements accurate to 0.2 cm.
‡ Measurements accurate to 10 micra.
§ Average based on 6 rats.

TABLE 2. GROWTH RETARDATION FROM FOOD RESTRICTION FOR 30 DAYS IN NORMAL 26 DAY OLD MALE RATS

| Treatment | No. of Rats | Final Body Weight | Body Weight Gain in 30 Days | Final Length Nose to Tail Tip | | Width of Proximal Epiphyseal Cartilage of Tibia | | Average Daily Food Intake |
|---|---|---|---|---|---|---|---|---|
| Restricted Food | 6 | *gm.* 181 (176–186) | *gm.* 111 (101–120) | *cm.* 34.8 (33.6–36.2) | *SE* ±0.36 | *micra* 352 (292–400) | *SE* ±9.8 | *gm.* 8.9 |
| Control | 6 | 244 (222–278) | 174 (152–205) | 38.3 (36.6–39.5) | ±0.46 | 445 (380–496) | ±17.9 | 12.8 |

of controls, (352 as compared with 445 micra) whereas in the ACTH treated group, the difference was 160 micra (249 as compared with 409 micra). The amount of juxta-epiphyseal endochondral bone was somewhat less in the food restricted group than in the control, but the change was not as marked as anticipated from the reduced overall measurements of the animals. It is evident therefore, that markedly reduced food consumption exerts less growth inhibition than does the injection of ACTH, the food consumption under the latter circumstances being normal.

The effects of food restriction on cartilage width and bone formation were certainly not so extreme as those resulting from hypophysectomy.

### COMPARISON OF THE EFFECTS OF ACTH ON GROWTH OF NORMAL AND ADRENALECTOMIZED RATS

The growth retardation in normal rats must have been induced by ACTH through its specific stimulation of the adrenal cortex, for ACTH had no effect on growth of the adrenalectomized rat (Evans, Li and Simpson, 1943). Growth of adrenalectomized rats (males 26 days of age) during injection of ACTH (1 mg. daily, divided in 3 injections) for a 30 day period was compared with that of untreated

TABLE 3. EFFECTS OF INJECTION OF ADRENOCORTICOTROPIC HORMONE* FOR 30 DAYS IN ADRENALECTOMIZED 26 DAY OLD MALE RATS (MAINTAINED ON 1% NaCl IN THE DRINKING WATER)

| Treatment | No. of Rats | Final Body Weight | Body Weight Gain in 30 Days | Final Length Nose to Tail Tip | | Width of Proximal Ephyseal Cartilage of Tibia | | Average Daily Food Intake |
|---|---|---|---|---|---|---|---|---|
| | | *gm.* | *gm.* | *cm.* | *SE* | *micra* | *SE* | *gm.* |
| ACTH | 8 | 182 (147–210) | 108 (73–150) | 36.3 (35.2–37.5) | ±0.21 | 294 (188–400) | ±23.3 | 11.5 |
| Control | 9 | 186 (152–216) | 114 (82–157) | 37.1 (35.3–38.9) | ±0.37 | 294 (198–376) | ±17.3 | 11.9 |

adrenalectomized controls. All rats were maintained from the time of operation on drinking water containing 1 per cent NaCl. It will be noted in Table 3 that after adrenalectomy, growth in body weight and length was not significantly less in injected than in uninjected rats, indicating that the action of ACTH in normal rats was through the adrenal cortex. Examination of the tibias of ACTH-injected adrenalectomized rats showed no histological difference from their controls.

### CONCLUSIONS

1). The treatment of normal rats with ACTH resulted in a retardation in both chondrogenesis and osteogenesis in the region of the proximal epiphysis of the tibia. The reduction in width of the epiphyseal cartilage while constant, was not so extreme as that following hypophysectomy. Furthermore, in hypophysectomized rats, osteogenesis ceased, whereas in ACTH treated rats it continued at a reduced rate.

2). Significant food restriction resulted in a retardation of body weight gain and bone growth which was somewhat less marked than that following ACTH injection when the food consumption was approximately normal. It is evident that lessened food consumption could not explain the effects of ACTH.

3). Growth inhibition and bone changes were not caused by ACTH in the absence of the adrenals.

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# ANTAGONISM OF PITUITARY ADRENOCORTICOTROPIC HORMONE (ACTH) TO THE ACTION OF GROWTH HORMONE ON THE OSSEOUS SYSTEM OF HYPOPHYSECTOMIZED RATS[1]

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A REPORT HAS already been made on the inhibiting effect of pure adrenocorticotropic hormone on the body weight increment of *normal* rapidly growing, young male rats (Evans, Simpson and Li, 1943). The effects on bone growth in these animals have also been described: retardation of both chondrogenesis and osteogenesis was involved. (See preceding paper).

Furthermore, the antagonistic action of ACTH on the body growth of *hypophysectomized* rats injected with growth hormone has recently been reported by Marx, Simpson, Li and Evans (1943). The increments in body weight and in the width of the epiphyseal cartilages which are usually induced by growth hormone were inhibited completely, or almost completely, by simultaneous administration of ACTH. Histological analysis has been made of the proximal ends of the tibias of all animals, a total of 108 rats, but a single typical series is described here.

Female rats, 26 to 28 days of age, were hypophysectomized and a two-week period was allowed to elapse before onset of the experiment. Eight rats were injected daily intraperitoneally with 0.1 mg. growth hormone for a fifteen-day period (13 injections, a total of 1.3 mg. being given). Three rats received ACTH intraperitoneally, 0.05 mg. twice daily or a total of 1.3 mg. Eight rats received both substances while seven were held as uninjected controls. The irregularity of the numbers of animals in the different groups was due to elimination of animals by sickness or death, or to exclusion because of incompleteness of the hypophysectomy.

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The body weight of the animals was followed carefully: the uninjected hypophysectomized controls lost 5 gm. during the 15 day period; the group receiving ACTH also lost weight (4 gm.); those receiving growth hormone gained 16 gm.; those receiving the combination gained only 8 gm. The food intake was not measured in this series, but it may be noted, by consulting the previous paper (Marx, Simpson, Li and Evans, 1943, Table 3, p. 104) that the food consumption of the hypophysectomized rats receiving much higher doses of ACTH, either alone or in combination with growth hormone was equal to, if not greater than, that of rats receiving only growth hormone; the controls ate the least of any group. Histological examination of the thyroids and ovaries showed no stimulation of these organs in any injected group.[2] The adrenals were stimulated in all groups receiving ACTH. The average weight of the adrenals in the respective groups was: control 12 mg.; ACTH 21 mg.; growth hormone 14 mg.; combination 19 mg.

The proximal ends of the tibias were sectioned and stained with hematoxylin and eosin. Figures 1 to 4 illustrate the typical results in the region of the proximal epiphysis. Figure 1 shows the narrow epiphyseal cartilage in the hypophysectomized untreated control (average for group, 253$\mu$). The cartilage columns were regular and parallel to the long axis of the shaft. The zone of vacuolated cartilage was reduced to a few cell layers. Only a few coarse bony trabeculae were still in direct contact with the cartilage columns (see Fig. 5). Such trabeculae included the cartilaginous matrix (stained blue) in center of the red stained bony trabeculae. Active osteoblasts were absent from the surfaces of these trabeculae. In the region between the bony trabeculae, the marrow abutted directly upon the cartilage cells and capillaries were infrequent. The sub-periosteal osteoblasts, on the other hand, appeared active. Figures 2 and 6 show that the state of the cartilage and of bone formation was essentially the same in the hypophysectomized rat receiving ACTH. The cartilage cells in the zone of erosion and the trabeculae of bone were, however, not as regularly arranged as in the control. Perhaps a few more capillaries

[2] The ACTH and growth hormone preparations used did not, in fact, show these contaminants at 5 to 10 times the total doses used here.

PLATE I. Proximal epiphyseal cartilage of right tibia of rat, hypophysectomized 26th day; 56 days of age at autopsy. Central sagittal section. Hematoxylin and eosin, 8 micra, ×50.

FIG. 1. Untreated control. (Spec. 8014, Pl. 7983).

FIG. 2. Injection with 0.1 mg. ACTH daily for 15 days, total amount 1.3 mg. (Spec. 8035, Pl. 8114).

FIG. 3. Injection with 0.1 mg. growth hormone daily, total amount 1.3 mg. (Spec. 8027, Pl. 7985).

FIG. 4. Injection with growth hormone and ACTH, doses as above. (Spec. 8021, Pl. 7986).

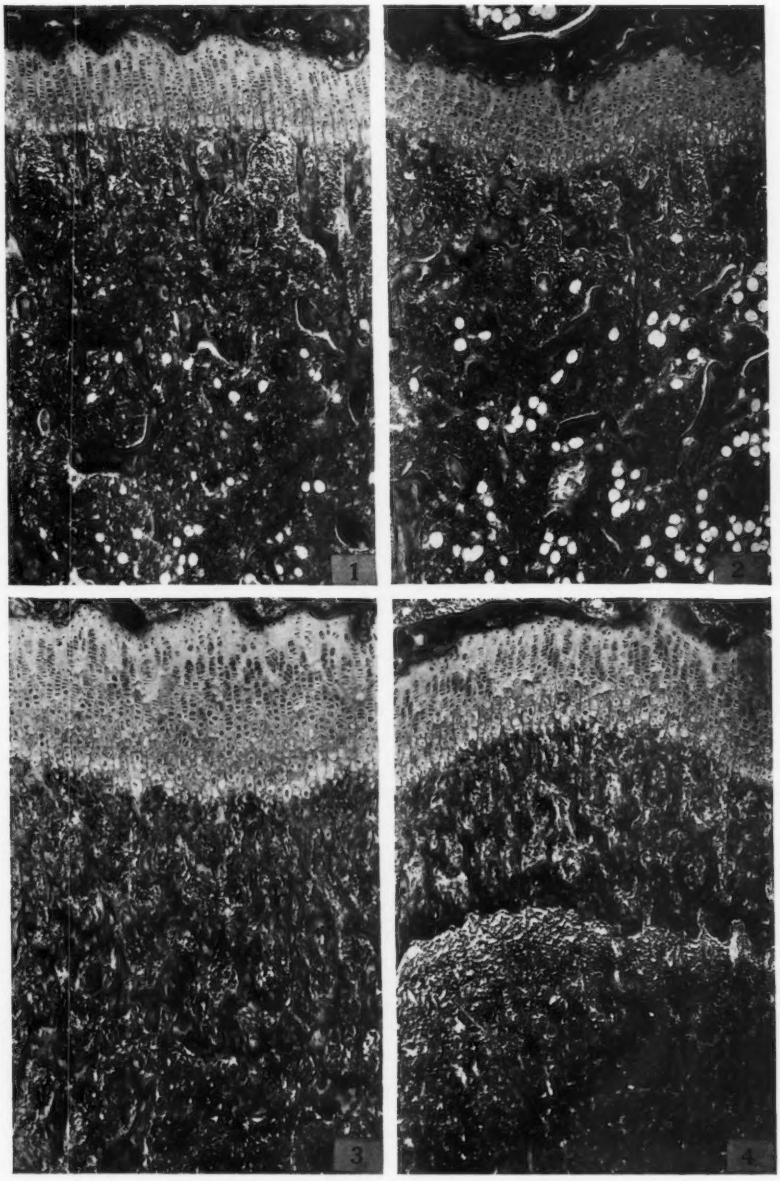


PLATE I. See opposite page for explanation.

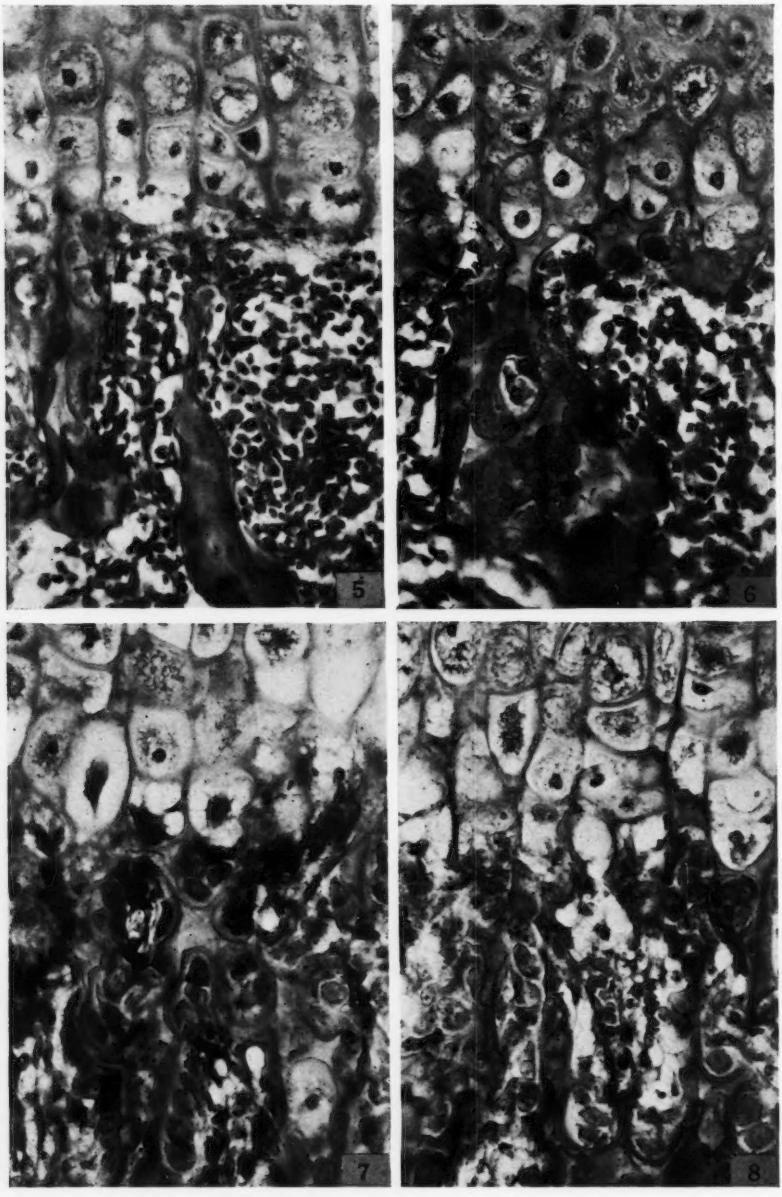


PLATE II. See opposite page for explanation.

from the marrow were touching the cartilage, but here, as in the control, very few osteoblasts were active in this zone. The marrow contained more fat cells than in the control (Figures 1 and 2). The epiphyseal cartilage was not significantly different (average 26 micra wider[3] than in the control with an accuracy in measurement of 10 micra).

Figures 3 and 7 show the marked stimulating effects of the growth hormone administered. The epiphyseal cartilage was much wider, the cells were larger, and the zone of vacuolated cartilage cells was distinct. The mean width of cartilage in the group was 393$\mu$, an increase of 140$\mu$ above control average. Osteogenesis was going on rapidly; regular erosion of cartilage cells was occurring, capillaries directed against the cartilage cells were numerous, and osteoblasts were present in the immediate juxta-epiphyseal zone, where they were being converted into osteocytes incased in bone on the surface of the residual cartilaginous matrix. The trabeculae of bone were arranged in orderly rows and extended far into the marrow. Active bone formation on these trabeculae deep in the marrow was shown by the large numbers of osteoblasts on their surfaces. The marrow was compact, fat cells being practically absent. (This confirms previous observations: Ray, Evans and Becks, 1941). Figure 4 shows the effect of injecting ACTH simultaneously with the growth hormone. It will be seen that the width of the cartilage was much less than with growth hormone alone. The vacuolated cartilage cells were, however, somewhat larger than in the control; the extensive degeneration of these cells, as characterized by the large non-nucleated cells in the erosion zone in animals receiving growth hormone, was not present (Figure 8). Capillary invasion could be seen and osteoblasts were present in the erosion zone. In contrast to the condition in rats receiving growth hormone, where the bony trabeculae extended in regular formation far into the shaft, here they were crowded and irregular[4] and ended abruptly a

[3] In the first paper on this subject (Marx, Simpson and Evans, 1943) the epiphyseal cartilage in ACTH treated groups was reported to be narrower than in control groups. There, the measurements were made from the split tibia fixed in formol and stained with silver nitrate. Only the uncalcified cartilage remained unstained by the silver and was measured. Here, in measurements made from hematoxylin and eosin stained preparations, calcified cartilage was also included in the width of the epiphyseal disc.

[4] The same irregular arrangement of bony trabeculae was observed in normal rats treated with ACTH and is illustrated in the paper dealing with this subject (see preceding paper).

PLATE II. Magnifications of juxta-epiphyseal regions shown in Figs. 1 to 4. Hematoxylin and eosin, 8 micra, ×205.

FIG. 5. Untreated control. (Spec. 8014, Pl. 7987).

FIG. 6. Injection with 0.1 mg. ACTH daily for 15 days, total amount 1.3 mg. (Spec. 8035, Pl. 7973).

FIG. 7. Injection with 0.1 mg. growth hormone daily, total amount 1.3 mg. (Spec. 8027, Pl. 7974).

FIG. 8. Injection with growth hormone and ACTH, doses as above. (Spec. 8021, Pl. 7980).

short distance from the cartilage. Since in the fourteen days following hypophysectomy osteogenesis ceased and shaft trabeculae were resorbed, the trabeculae present here represent those formed under the influence of the simultaneous administration of growth hormone and ACTH and are very considerable albeit less than when growth hormone alone was administered. Both osteoblasts and osteoclasts were practically absent at this abrupt ending of the bony trabeculae. The marrow was very cellular.

## CONCLUSIONS

Comparisons have been made of the proximal epiphyseal regions of the tibia of rats 29 days after hypophysectomy when uninjected, and when injected with ACTH, with growth hormone or with the combination.

ACTH given alone scarcely affected the inactive condition of the epiphysis, except that the cartilage cells in the erosion zone were more irregular. Statis of bone formation was characteristic, just as in controls.

Growth hormone activated the cartilage and caused resumption of formation of delicate, straight trabeculae of bone.

As a result of simultaneous administration of ACTH and growth hormone, the following changes in the typical effects of growth hormone were observed: a) The proximal epiphyseal cartilage of the tibia was greatly decreased in width. b) Endochondral bone formation was significantly retarded. c) Osteoblastic as well as osteoclastic activity was greatly decreased, perhaps accounting for the irregular arrangement of bony trabeculae. d) The cartilage columns in the erosion zone were also more irregular.

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# ANATOMICAL EFFECTS OF THIOURACIL

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THE GOITERIGENIC effect in rats of a large number of substances related to thiourea has been demonstrated by Richter and Clisby (1942), Kennedy (1942), Astwood and co-workers (1943), and by MacKenzie and MacKenzie (1943). The latter two groups of investigators showed that these compounds inhibit the production of the thyroid hormone and bring about a decreased basal oxygen consumption, increased pituitary activity, and hyperplasia of the thyroid gland. Astwood (1943 a) found that 2-thiouracil was more goiterigenic than any one of a large number of thioureas, sulfonamides and other substances which he tested.

Astwood (1943 b) found that when thiouracil was administered to thyrotoxic patients for several weeks there resulted a clinical remission of the disease with a return of the basal metabolic rate to a normal range. Williams and Bissell (1943) confirmed these observations and also found that the protein bound iodine of the plasma returned to normal levels after a few weeks of treatment. However, during the course of these clinical studies a few patients were found to develop edema, the etiology of which may possibly have been due to alterations in adrenal function (Williams, Bissell, Jandorf, and Kay, unpublished). Thiouracil has also been found to cause skin rashes, agranulocytosis, headache, fever and perhaps other complications. The drug has been found to distribute itself throughout essentially all of the tissues and fluids in the body (Williams, Kay and Jandorf, 1943), establishing a relatively high concentration in the pituitary, adrenal, thyroid, ovary and bone marrow. With these considerations in mind it seemed to us that a study of the effects of thiouracil on the structure of the various tissues of the body was indicated. Such a study might reveal the possibility of unexpected complications, or, on the other hand, suggest a usefulness of the drug in the treatment of diseases other than thyrotoxicosis. Consequently, we undertook an investigation of the anatomical effects of thiouracil when it is administered to normal animals and when it is given to rats also receiving thyrotropic,

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adrenotropic, gonadotropic or growth hormones. The results of these studies we are reporting in this paper.

## METHODS

All of the animals used were white Wistar rats which we had raised and were in excellent health. More than 200 rats with weights ranging from 14 to 325 gm. were studied (table 1). These animals were divided into 18 series, consisting of from 4 to 24 rats in each; these were sub-divided into about equal-sized groups, some of which were normal and others treated in the manner described later. In each series the animals were either litter-mates or

TABLE 1. EFFECT OF THIOURACIL ON THE SIZE OF THE PITUITARY, GONADS AND ADRENALS OF RATS

| Series | Total Rats | Average Weight Before Treatment gm. | Days of Thiouracil Treatment | Weight of Glands (mgm. per 100 gm. body weight) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Pituitary | | Testes or Ovaries* | | Adrenals | | | |
| | | | | Normal | Thiouracil | Normal | Thiouracil | Normal | Thiouracil | Adrenotropic hormone | Adrenotropic hormone + thiouracil |
| 1 | 16 | 300 | 27 | 2.6 | 2.9 | 1070 | 890 | 13 | 9 | 14(13-15) | 17(14-22) |
| 1A | 4 | 325 | 60 | 2.9 | 2.5 | | | 9 | 11 | | |
| 2 | 16 | 40 | 7 | | | | | 25 | 19 | 35(33-37) | 47(38-55) |
| 2A | 16 | 65 | 7 | | | | | 27 | 20 | 29(22-38) | 29(27-32) |
| 3 | 8 | 240 | 12 | 3.6 | 3.1 | 1320 | 1080 | 18 | 15 | | |
| 4 | 16 | 200 | 22 | 2.9 | 3.3 | 1160 | 1510 | 15 | 18 | | |
| 5 | 10 | 45 | 10 | 3.1 | 4.9 | 23 | 32 | 19 | 30 | | |
| 5A | 6 | 45 | 10 | 3.5 | 3.5 | 2160 | 2430 | 21 | 21 | | |
| 6 | 8 | 75 | 21 | | | 39 | 33 | 24 | 20 | | |
| 7 | 6 | 105 | 14 | 4.3 | 3.2 | 1750 | 1740 | 18 | 17 | | |
| 8 | 12 | 30 | 14 | 4.1 | 5.0 | 45 | 32 | 15 | 21 | 24(23-25) | 30(26-36) |
| 9 | 8 | 30 | 20 | | | 740 | 420 | 18 | 21 | | |
| 12 | 8 | 21 | 16 | 2.6 | 1.9 | 689 | 396 | 21 | 21 | | |
| 14 | 9 | 14 | 11 | 4.6 | 3.7 | 21 | 19 | 21 | 22 | | |
| 15 | 11 | 95 | 12 | 3.1 | 3.1 | 26 | 28 | 20 | 17 | | |

* Small weights are ovaries.

born within 2 days of each other and usually were of the same sex. Rarely was there more than 10 per cent deviation in the body weight in any series at the beginning of the experiment.

Thiouracil[1] was administered in the drinking water as a 0.25 per cent solution in the form of its sodium salt, the pH of the solution being about 8.5. A fresh supply was given every 2 or 3 days. The pituitary hormones were administered subcutaneously, and the site of the injection was changed each time.

The animals were killed by means of a blow on the head. In many of the animals (table 1) we rapidly dissected, under a microscope, the adrenals, gonads, thyroid and pituitary. Each gland was immediately dropped into olive oil[2] and weighed on a microbalance. This procedure was performed by 2 people, one determining the weights while the other dissected. It usually required only 1 to 2 minutes to dissect each gland. In almost all of the treated animals an examination was made of the gross appearance of the skeletal muscle, kidneys, spleen, pancreas, intestines, thymus, heart, lungs, brain, and the vertebral bone marrow. In about one-third of the normal animals

[1] The thiouracil was supplied by the Lederle Laboratories, Inc., Pearl River, N. Y.

[2] The tissues were weighed in oil in an effort to help avoid loss of water vapor.

similar examinations were conducted. From some of the animals in each of the 18 series described above sections were taken from essentially all the tissues of the body. A total of more than 700 microscopic sections were prepared; there were many more sections of the endocrine glands and of the bone marrow than of the other tissues. These were fixed in Zenker's solution, mounted in paraffin and stained with eosin and methylene blue. All of the sections were studied by one of us (R. H. W.) and representative ones were examined by Dr. F. Parker, Jr.

From a few rats, at the time of death, we obtained samples of blood for the estimation of the content of thiouracil, using the method of Williams,

TABLE 2. EFFECT OF THIOURACIL ON THE BLOOD CELLS

| Duration of Treatment | Blood Thiouracil mgm./100 cc. | Hemoglobin gm. | White Blood Cells |
|---|---|---|---|
| 3 mos. | 9.6 | 6.5 | 5,600 |
| 3 mos. | 8.8 | 8.0 | 10,500 |
| 3 mos. | 10.2 | 7.0 | 4,400 |
| 1 mo. | 1.2 | 8.5 | 9,200 |
| 1 mo. | 2.4 | 14.0 | 4,250 |
| 1 mo. | 2.0 | 8.0 | 6,750 |
| *10 days | 9 | | 3,600 |
| *16 days | 6.0 | 6.5 | 7,000 |
| *12 days | 5.6 | 9.5 | 19,100 |
| Normal (25 rats) | Aver. | 13 | * 9,000 |
| | Range | 10–16 | 6,000–19,000 |

* The values were obtained from the pooled blood of 3 rats.

Jandorf and Kay (1943). In some rats we determined the white blood cell counts and the hemoglobin content (table 2).

## RESULTS

(a) *Body Growth.* The rate of growth as measured by weight gain, was followed in 11 series of animals, totaling 88 rats, the initial weights of which varied from 14 to 105 gm. Forty-five animals were treated with thiouracil for an interval of from 7 to 21 days. The average growth rate was distinctly less than normal in each of the series of animals receiving thiouracil for 10 days or longer (figure 1). In 4 series of animals we not only studied the effect of thiouracil on normal growth, but also the effect of the drug on animals simultaneously treated with growth hormone. Sixteen rats of essentially the same size were divided into 4 groups of equal size (series 2). One group received no treatment, one thiouracil, one "growth hormone"[3] and one thiouracil plus "growth hormone." The thiouracil was administered

[3] This "growth hormone" consisted of the small amount present (10 rat units per cc.) in Armour's Adrenotropic Factor. We use quotation marks to differentiate this preparation from another one which we used, the Growth Complex, which has 10 times as much growth hormone.

for 7 days, and the "growth hormone" was given concomitantly during the last 5 days, the dosage being 0.5 cc. twice daily. When "growth hormone" alone was given the growth rate was above normal (figure 1, B), but when it was given with thiouracil the growth rate was distinctly subnormal. Exactly the same experiment was conducted with the animals in series 2a, the only difference being that the initial weight of these animals was 65 gm. rather than 40 gm. Comparable results were obtained in the 2 experiments. The study

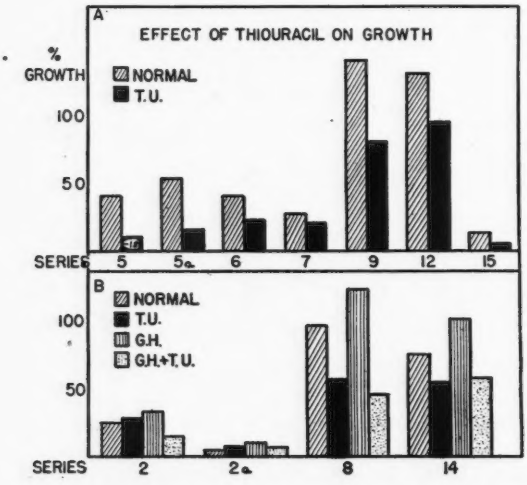


FIG. 1. Each series consisted of rats which were of the same size before treatment with thiouracil. However, the size of the animals and the duration of treatment varied from series to series, as explained in the text and table 1.

The percentage of growth represents the amount of weight gained during the course of the experiment, as compared to the initial weight of the animal. This was determined in the case of each animal and then the average rate of growth of the series was computed.

Note that thiouracil not only inhibits normal growth (A) but also abolishes the effect of the growth hormone (B).

was again repeated using younger rats, a more potent hormone preparation, and longer treatment. These animals (series 8) were given thiouracil for 14 days with 0.5 cc. daily of growth hormone (Growth Complex[4]) during the last 12 days. The results are well-defined[5] and

[4] The Growth Complex was supplied by Ayerst, McKenna and Harrison, Rouses Point, N. Y. It has 100 rat units (Collip) of growth factor and 10 rat units (Collip) of adrenotropic factor per 1 cc.

[5] In series 8 the gain in weight in the normal group was from 91 to 100 per cent; in the group treated with growth hormone it was from 116 to 128 per cent; in the group treated with thiouracil it was from 44 to 70 per cent; and in the group treated with growth hormone and thiouracil it was from 36 to 59 per cent.

show that thiouracil not only retards normal growth but also abolishes the effects of the growth hormone (figure 1). The experiment conducted with the animals in series 14 is unique in that the only source of supply of thiouracil was in the mother's milk. Williams, Kay and Jandorf (1943), found that human milk contained much more thiouracil than did any other body fluid which they examined. Two rats, one with a litter of 9 and the other with a litter of 10, were used for study. The rats of each litter were exactly the same age and weighed 14 gm. ±0.5 gm. Five rats from each litter were exchanged. One mother was

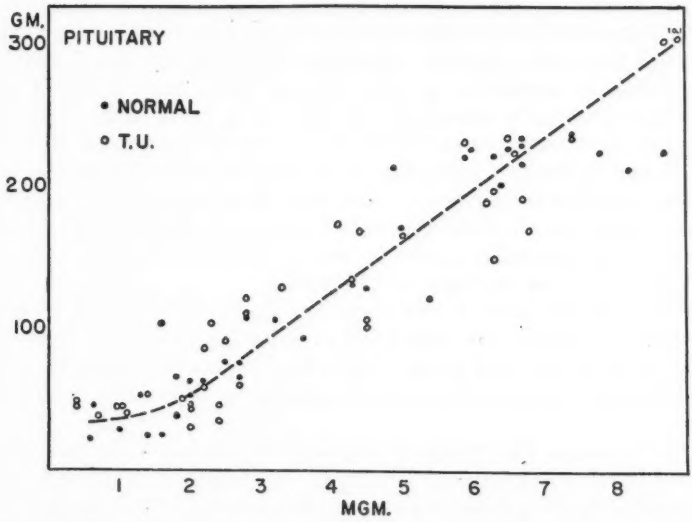


FIG. 2. The weight of each rat pituitary is plotted in relation to the body weight of the animal. It can be observed that the ratio of pituitary weight to body weight is the same in the animals treated with thiouracil (T. U.) as in normal rats of the same size.

given thiouracil in the drinking water while the other one had none of this drug. Five infant rats in each cage were injected with growth hormone for 9 days, beginning at the same time that the thiouracil treatment was started. Polyansyn[6] was given for 3 days in doses of 0.1 cc. twice daily. Growth hormone[4] was then administered for 6 days in doses of 0.1 cc. twice daily, and 2 days after discontinuing the injections the animals were killed. Samples of blood from 3 of the thiouracil-treated young rats were pooled and an analysis of the thiouracil content showed a concentration of 5.6 mgm. per 100 cc.; the concen-

[6] Polyansyn was supplied to us by The Armour Laboratories, Chicago, Illinois. It contains 25 growth units (Collip) in 1 cc.

The many changes in the type of growth hormone used were due chiefly to the present scarcity of this product.

tration of this drug in the mother's blood was 12 mgm. per 100 cc. Therefore we know that the young rats received the drug in fairly large quantities. Furthermore, as one may observe in figure 1, the normal growth was inhibited by thiouracil, and the effects of the growth hormone were abolished.[7]

The retardation in growth produced by thiouracil was observed not only as regards the body weight but also body length and size of the organs. We have noticed that the adrenals and gonads of the thiouracil-treated animals almost always weigh less than those of their normal siblings.

The inhibitory effects of thiouracil on growth may be related to its inhibition of the production of thyroid hormone. It has been suggested that this hormone normally stimulates the pituitary gland which, in turn, tends to accelerate growth (Flower and Evans, 1924). By the time that a definite retardation in growth is induced by thiouracil, about 10 days, enlargement of the thyroid is also observed. However, Bissell and Williams (1943) found no definite alteration in the basal oxygen consumption of rats treated with thiouracil for this interval.

Astwood and co-workers (1943) found that the growth-retarding effects of sulfaguanidine could be largely abolished by the simultaneous administration of desiccated thyroid.

However, the effect which thiouracil exerts on the thyroid would not seem to explain its inhibition of injections of the growth hormone, since Flower and Evans (1924) have shown, in young rats, that this hormone can maintain normal growth in thyroidectomized animals.

(b) *Pituitary.* The weight of the pituitary gland in the animals receiving thiouracil treatment tended to be smaller than normal. However, when one compares the size of the gland in proportion to body weight one does not observe any definite difference in the 2 groups (table 1, figure 2). Microscopic examination of many sections of the pituitary of animals fed thiouracil for intervals of from 7 to 60 days failed to show any alteration in the structure. This was true not only of tissue stained with eosin and methylene blue but also of some stained with Mallory's aniline blue. Changes in the pituitary similar to those occurring after thyroidectomy have been described by some authors as following the use of certain sulfonamides and thiourea (MacKenzie and MacKenzie, 1943).

(c) *Adrenals.* We were particularly interested in ascertaining whether this drug exerted an effect on the adrenals, because of the possible utilization of such a drug in the treatment of hyperadrenocorticism. The adrenals of the animals treated with thiouracil were

[7] In series 14 the gain in weight in the normal group was from 62 to 74 per cent; in the group treated with growth hormone it was from 90 to 114 per cent; in the group treated with thiouracil it was from 40 to 61 per cent, and in the group treated with growth hormone and thiouracil it was from 30 to 71 per cent.

distinctly smaller than those of their siblings. However, if one calculates the weight of these glands in proportion to the body weight no significant difference is found (table 1). This suggests that the absolute variation in the size of the adrenals in the 2 groups was probably related to the retardation in the body growth of the treated animals. However, the weight of the adrenals does not increase in direct proportion to the increase in weight. Because of this fact we extended our studies to include a large number of rats with many gradations in size. With this data we plotted the weight of the adrenals against the

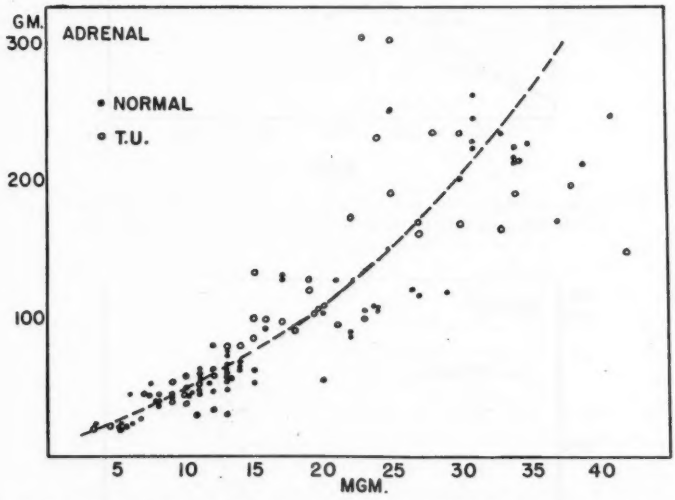


FIG. 3. The ratio, weight of the adrenals to body weight, is the same in the animals which received thiouracil as in normal rats of the same size.

body weight of the animal and obtained a curve (figure 3) which reveals no definite difference in the size of the adrenals of the thiouracil-treated animals from the normal animals of the same weight.

The effects of thiouracil on the adrenomegalic properties of adrenotropic hormone were also studied in 4 series of animals. The adrenotropic hormone therapy given to series 2, 2a and 8 was discussed in the section on Body Growth. The same amount of hormone therapy was given to the animals in series 1 as to the ones in series 2 and 2a but thiouracil treatment had been given over a longer interval before the hormone therapy was started. The weights of the adrenals of the animals receiving thiouracil plus adrenotropic hormone tended to be somewhat larger than those receiving only the latter (table 1).

Microscopic examination of a large number of sections of the adrenal glands of animals treated with thiouracil failed to show any alterations in the normal structure. This was true not only of the

sections stained with eosin and methylene blue but also those stained with Sudan IV.

(d) *Gonads.* The testes and ovaries of the thiouracil-treated animals were smaller than were those of the normal controls, the difference being presumably due to the retardation in the body growth of

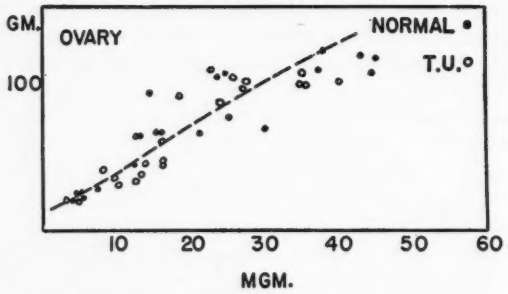


FIG. 4. The ratio, weight of the ovaries to body weight, is the same in the animals which received thiouracil as in normal rats of the same size.

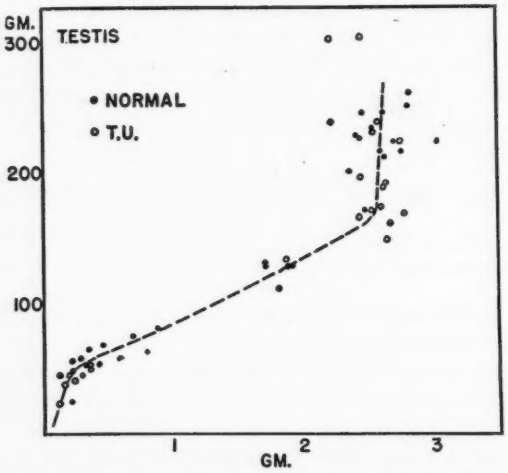


FIG. 5. The ratio, weight of the testes to body weight, is the same in the animals which received thiouracil as in normal rats of the same size.

the treated animals. However, in the case of many animals when the weight of the gonads is plotted against the body weight a curve is produced which shows no difference in the size of the glands in the 2 groups (figures 4 and 5). No microscopic alterations were found to be induced by thiouracil in either the testes or ovaries.

We also studied the effects of thiouracil in inhibiting the effects of

pregnancy urine. Each of 8 female rats, 21 days old, was injected with 1 cc. of human pregnancy urine twice daily for 2.5 days. An assay of this urine had shown that each cc. exerted an effect on normal immature female rats equivalent to one rat unit of chorionic gonadotropin. On the day before the injections were started 4 of these animals were given thiouracil in the drinking water. This was continued for 6 days, and then all animals, including 4 normal controls, were killed and microscopic sections were made of the ovaries. Thiouracil was not found to interfere with the formation of follicles or corpora lutea in the ovaries.

In another experiment of a similar nature, a preparation of chorionic gonadotropin[8] was used. Each of 8 female rats, weighing 40 grams, was given subcutaneously 0.1 cc. of the solution daily for 5 days. Four of these animals had a 0.25 per cent solution of thiouracil as their only source of drinking water beginning one day before injections of the hormone were begun, whereas the other 4 had tap water. On the sixth day all animals were killed. Follicles and corpora lutea were found in each group in approximately equal numbers.

(e) *Thyroid.* The marked goiterigenic effects of thiouracil described by Astwood and associates (1943) were fully confirmed. We also observed the marked cellular hyperplasia and decrease in colloid which they described. The glands of rats treated for several weeks showed very small amounts of colloid. The acinar arrangement of the cells was sometimes replaced by solid sheets of cells. Distinct thyroid enlargement was exemplified in intervals as short as 7 days (figure 6). The changes were present in all age groups. It was of interest to note that enough thiouracil was transmitted in the milk of a rat to promote marked enlargement of the thyroid of infant rats (series 12, figure 6).

In one experiment we compared the goiterigenic effect of thiouracil to the effect of thyrotropic hormone. We also studied the changes produced in the thyroid by the simultaneous administration of these 2 substances with and without potassium iodide. All of the thyrotropic hormone[9] was given subcutaneously in doses of 0.1 cc. daily. The potassium iodide was given in the drinking water as a 50 mgm. per cent solution. Thiouracil was given as its sodium salt in a 0.25 per cent solution. When potassium iodide and thiouracil were given to the same animals they were given as one solution in the concentrations just mentioned. For this experiment we used 24 rats (series 11), of the same age and weighing approximately 70 gm. They were divided into 6 groups of 4 rats each and were treated for 10 days as

[8] A. P. L. (anterior pituitary-like substance) was supplied by Ayerst, McKenna and Harrison, Rouses Point, New York. It contains 500 I. U. of gonadotropic factor in 1 cc.

[9] The thyrotropic hormone (antuitrin-T) was supplied by Parke Davis & Co., Detroit, Michigan. Each cc. of this preparation contains approximately 50 Junkmann-Scholler units.

follows: (a) no treatment, (b) thyrotropin, (c) potassium iodide and thyrotropin, (d) thiouracil, (e) thiouracil and thyrotropin, (f) thiouracil, thyrotropin and potassium iodide. The weights of the thyroids in the different groups, expressed in terms of mgm. per 100 gm. of body weight were as follows: normal 7 to 9 (mean 8.4); thiouracil 11 to 19 (mean 16); thyrotropin 11 to 15 (mean 13); thiouracil plus thyrotropin 15 to 20 (mean 19); thyrotropin plus potassium iodide 10 to 18

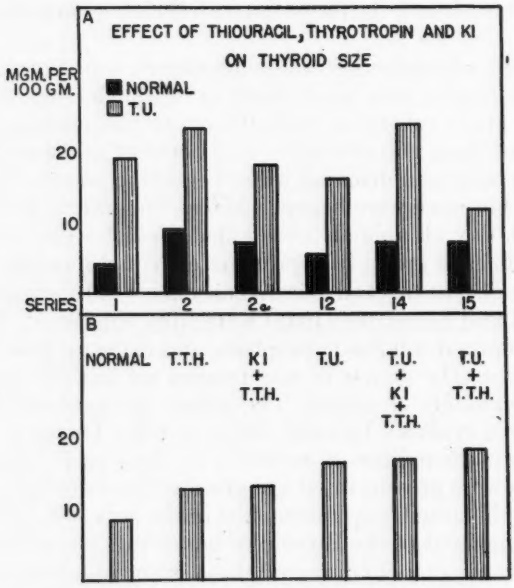


FIG. 6. The animals in each series were essentially of the same size at the beginning of treatment. However, there was variation in size and in the duration of treatment in the different series. The thyroid gland from each animal was weighed and its weight expressed as mgm. per 100 gm. body weight. These figures were averaged and plotted in the columns above. The results shown in the lower half of the chart were obtained from series 11.

Note the marked goiterigenic effect of thiouracil (A). With the dosages used the thyrotropic hormone (T. T. H.) was not as goiterigenic as thiouracil (B) but the combined effect of the 2 substances was very slightly greater than that of either one singly. Potassium iodide (KI) had no definite effect on the goiterigenic activity of thyrotropin.

(mean 13); thiouracil, potassium iodide and thyrotropin 16 to 18 (mean 16).

The amount of hyperplasia found microscopically in the various groups was in accord with the increase in weight of the gland. However, this was not true of the amount of colloid. On the basis of decreasing contents of colloid the various groups were graded as follows: (1) normal, (2) thyrotropin and potassium iodide, (3) thyrotropin, potassium

iodide and thiouracil, (4) thiouracil, (5) thyrotropin, and (6) thyrotropin and thiouracil.

(f) *Hemopoietic System.* Since the most dreaded complication encountered from thiouracil is agranulocytosis we were very much interested in studying the effects of this drug when given in large doses, such as we have described above. Microscopic examination of the bone marrow of approximately 50 rats was made. These animals had received treatment for intervals of from 7 to 90 days. No definite abnormalities were found in any section.

A few studies were made of the circulating blood. Examinations were made on specimens obtained from the heart. In a few instances the total white count was low (table 2). The hemoglobin tended to be somewhat low, but the icteric index was normal.

Estimations of the thiouracil content of the blood, using the method of Williams, Jandorf and Kay (1943) were made 17 times, using the blood of 26 rats. In most instances there was from 6 to 10 mgm. per 100 cc. of blood. This level is distinctly higher than that obtained in patients receiving 1.2 gm. of thiouracil daily.

(g) *Other Tissues.* Microscopic examination was made of many tissues of approximately 25 rats which were treated with thiouracil for 7 to 60 days. In addition to the tissues previously mentioned this study included sections of skin, skeletal muscle, heart, lungs, thymus, pancreas, spleen, liver, kidneys, intestines, lymph nodes, brain and spinal cord. No effects of thiouracil were seen in any of these structures.

(h) *Human Tissues.* Six patients carrying a hopeless prognosis were given about 0.6 gm. daily of thiouracil for an interval of from 6 to 28 days. Postmortem examinations were conducted in each case and microscopic examination was made in all cases of the heart, lungs, spleen, liver, kidneys, adrenals, pancreas, gonads, thyroid and bone marrow. In 4 cases an examination was made of the brain and pituitary. No alteration in the structure of any of these organs was found which could be attributed to the use of thiouracil.

## SUMMARY

An investigation was made of the structural alterations produced in rats fed thiouracil in a relatively large amount for intervals of from 5 to 90 days. A study was also made of the inhibitory effects of this drug to the injection of various hormones; adrenotropic, thyrotropic, growth, and chorionic gonadotropin.

Thiouracil causes a distinct retardation in the rate of growth, the effect becoming apparent within a period of 10 days. It also tends to abolish the effects of injections of growth hormone. The impairment of growth seems to involve the entire body, excluding the thyroid. The pituitary, adrenals and gonads of thiouracil-treated animals were smaller than those of the siblings. However, the ratio of the weight of

these glands to the body weight was the same as in normal animals of equal body weight.

No inhibition of the effect of chorionic gonadotropin by thiouracil was demonstrated. The adrenomegalic activity of the adrenotropic hormone was augmented rather than inhibited, and the goiterigenic effects of the thyrotropic hormone were supplemented. Potassium iodide inhibited the colloid-depleting effect of thyrotropin on the thyroid whether thyrotropin was given alone or in conjunction with thiouracil.

In the limited study conducted a few animals showed a distinct leucopenia and anemia. However, the concentration of thiouracil in the blood of most of these animals was distinctly higher than we have encountered in man.

In more than 700 microscopic sections made from essentially all of the tissues of the body of many series of rats, no notable alterations were found which were attributable to thiouracil, with the exception of those in the thyroid gland. Six patients, who were given thiouracil for from 6 to 28 days preceding death, were not found on post-mortem examination to show any gross or microscopic effects of this drug.

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# THYMUS WEIGHT IN RELATION TO BODY WEIGHT IN CASTRATED AND IN ADRENALECTOMIZED RATS

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IT HAS BEEN observed in many species, that after puberty animals which have been castrated, adrenalectomized, or fed dessicated thyroid have larger thymus glands than their controls.

These findings led some of the investigators to designate the larger thymus in the experimental groups as "hyperplastic" glands. Others preferred to refer to failure or retardation of the physiological "age involution" under such conditions (Hammar, 1936).

It will be shown in this paper that this is a misconception. The thymus in such animals is neither un-involuted nor hyperplastic, but fails to undergo "accidental involution." This statement finds support in Selye's observation that the atrophy of the thymus in the "alarm reaction" does not occur in adrenalectomized animals (Selye, 1940).

The data on thymus weights presented below were obtained from male albino rats of the Sherman strain. The animals were killed and the thymus weighed on a microtorsion balance. The thymic parenchyma was determined in 20 mature rats (14 weeks of age) by planimeter measuring of camera lucida drawings of 4 sections from different levels of each gland. The interstitium was found not to exceed about 5% of the total weight of the gland.

Chart I presents a log./log. plot of thymus weight on body weight of animals from our stock colony on stock diet (Rockland pellets, D free) at various ages between birth and about 1 year. Growth records of these animals were not available.

Chart II gives the range of the data from Chart I as compared with data obtained from male albino rats on the same diet which had been castrated or adrenalectomized 3–4 weeks before killing. It shows also thymus weights with respect to body weight in animals which were not operated upon but weaned and maintained on 2 other diets: diet Z[1] and diet E[1]. It further includes castrated animals weaned on

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[1]*Diet E:* (used by Kaunitz, H., and A. M. Pappenheimer, Am. J. Phys. 138, 328, 1943.

Casein (commercial) 320 gms.; cornstarch, 400 gms.; salts (Hawk and Oser), 40 gms.; yeast (bakers dried) 100 gms.; lard 220 gms.; cod liver oil 20 gms. Alpha-tocopherol was given in weekly oral doses of 50 mgs.

*Diet Z:* (Zucker, T. F. and B. N. Berg, Proc. Soc. Exp. Biol. and Med. 53, 34, 1943) casein, 279 gms.; modified Wesson salts, 12 gms.; $CaCO_3$ 22.6 gms.; $KH_2PO_4$, 19.4 gms.; yeast extract 20 gms.; carotene, 1 gm.; wheat germ oil, 2.5 gms.; vitab, 50 gm.; riboflavin (30%), 10 cc.; cerelose to make 1000.

the diet E. In many of the animals in each group, the testes, adrenals, and thyroids were weighed and examined histologically. They were found to be normal.

It is seen from Chart II that in all of the 4 groups the range of the thymus weights with respect to body weight is higher than the range of weights in animals on the Rockland diet. Within these 4 groups they do not differ. The weights of other organs (pituitary, thyroids,

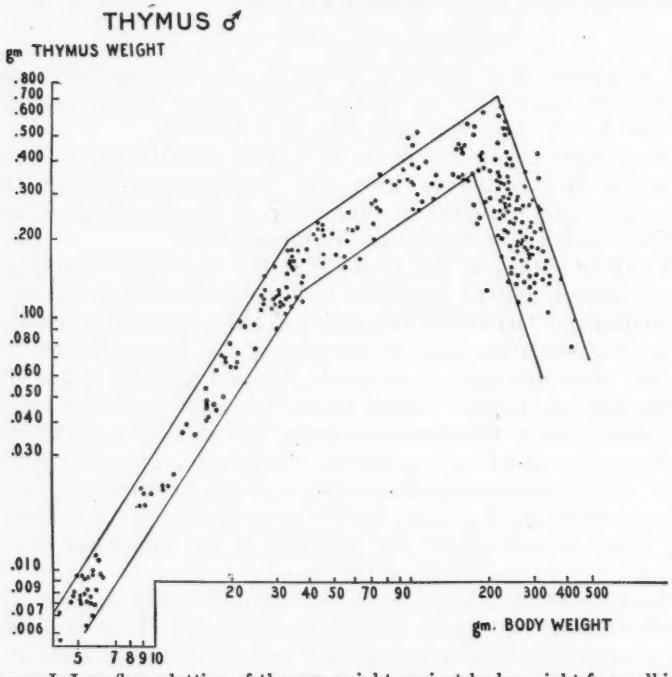


CHART I. Log./log. plotting of thymus weight against body weight from albino rats on Rockland diet.

adrenals, testes, spleen, and kidneys) did not differ among any of the 5 groups.

Thus, in accordance with previous reports, in castrated or adrenalectomized animals on the Rockland diet, we observed thymus glands of higher relative weight than that of animals which were not operated upon. This however, was true only when animals from our stock colony maintained on the Rockland diet were taken at random and considered as "normal controls." The thymus weights of animals on diet E, and on diet Z, whose testes and adrenals had not been removed, were found in the same weight range as those of castrated or adrenalectomized animals on the Rockland diet. Castration failed to produce a significant difference in the thymus size between experi-

mental animals and controls, when both groups were maintained on diet E.

In general, investigators have preferred to use age instead of body weight in order to correlate thymus weights when data over a range of animal life were given (Hammar, 1936). Huxley (1932), Needham (1942) and others have shown that a very simple relation for expressing relative growth is obtained if log. organ weight is compared with

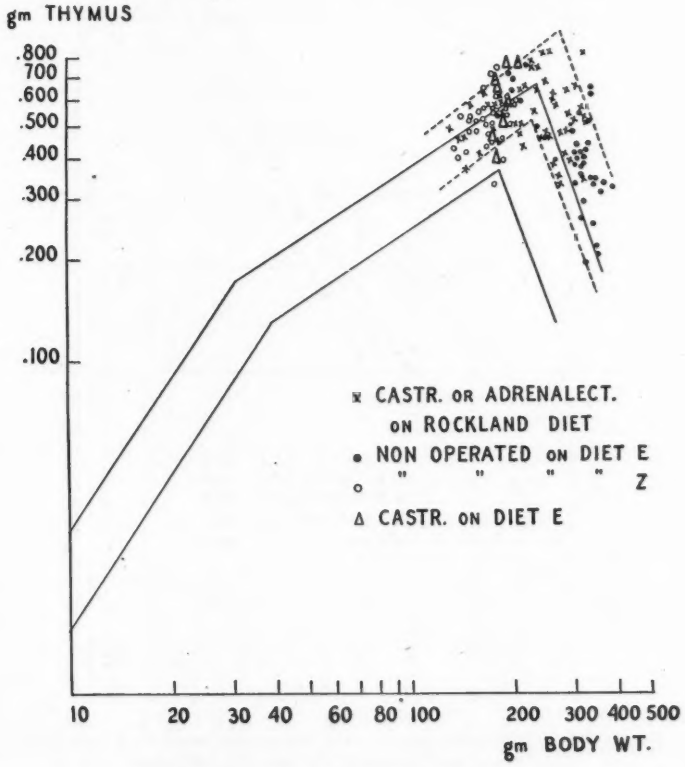


CHART II. Comparison of range of data from Chart I with experimental data.

log. body weight. The usual relation in a plot of this kind is a straight line. In many instances such heterogenies of organs or body constituents present one or several "breaks" (Needham, 1942; Zucker and Stoerk, unpublished observations).

It has been shown by Jackson (1939) that temporary growth retardation due to calorie restriction markedly affects thymus growth with respect to age but much less so with respect to body weight. Thus it appears that thymus weight is more closely correlated with body

weight than with age and that proper comparisons between experimental animals and their controls should be based on body weight.

In addition to a variety of stresses and strains, body weight loss due to starvation is known to be associated with a striking reduction in thymus size (Hammar, 1936). This unexplained phenomenon has been referred to as "accidental involution" of the thymus. It has been

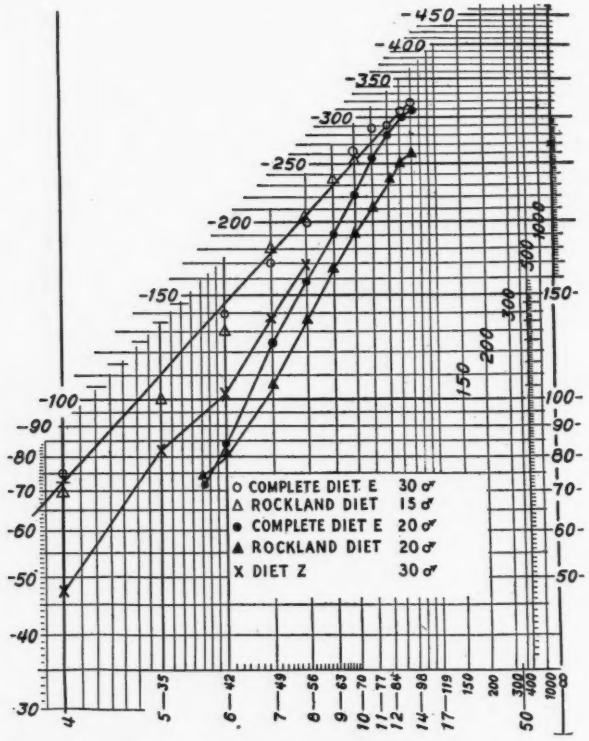


CHART III. Post weaning growth on a log weight—reciprocal time plot. The upper line represents the optimal male slope.

demonstrated by Jonson (1909) that this thymic atrophy is a reversible change. On realimentation of starved animals, restitution of the thymic parenchyma occurs.

If growing animals before the onset of the physiological "age involution" of the thymus are restricted in their food intake or are exposed to other adverse conditions, retardation of thymic development usually accompanies retardation of body growth. This retardation in thymic development in most instances is more pronounced than the retardation of body growth. The gland may actually atrophy. In post-

pubertal animals with body growth retardation, the "age involution" is usually accelerated (Stoerk, unpublished observations). As will be shown later, there are conditions in which retardation of body growth is not associated with atrophic changes in the thymus beyond the physiological regression. Such animals with retarded growth have a larger thymus than a normal growing animal of the same age. This does not imply that the larger glands are hyperplastic.

The tendency of the thymus gland to become reduced in size in response to a variety of suboptimal conditions must be taken in consideration when "normal" thymus weights are presented and used for comparison.

In view of these facts and in order to ascertain which set of data on thymus weights represents the "norm" for albinos of the Sherman strain, the following observations on growth of the animals in relation to the diets used in this investigation are presented.

Although on large numbers of healthy and well developed animals the Rockland rat diet was found to produce optimal growth (Zucker and Zucker, 1942) and excellent reproduction, we observed as seen from Chart III that animals from our colony taken from large litters and evidently undersized at the time of weaning showed considerably faster realimentation on the diet E than their littermates on the Rockland diet. The same was true for the diet Z. The thymus glands in the "faster growing" animals were larger than those of animals on the Rockland diet and were in the same range of relative weight as the thymus glands of operated animals on the Rockland diet or on the diet E. Selected animals from small litters with high weaning weights, kept on the Rockland diet, showed optimal growth, identical to animals with high weaning weights on the diet E. Their thymus weights were determined only in a few instances. They did not differ from the glands of animals on the diet Z or from that of operated animals.

In most colonies, unless special care is taken, a large number of animals is more or less undernourished at the time of weaning because of a very frequent discrepancy between the litter size and the nursing capacity of the mother. Respiratory infections may at any age lead to transitory growth retardation or weight loss. In view of these facts, diets like E and Z, which for unknown reasons permit more rapid realimentation, appear under the given circumstances superior to the Rockland diet, although in well developed, healthy rats, this diet also allows optimal growth.

It seems therefore that the set of thymus data from the animals on Rockland diet must be considered as obtained under suboptimal conditions. The larger thymus glands in the operated animals on the Rockland diet and diet E and in the intact animals on the diet E and Z as well as in the optimally growing animals on the Rockland diet most probably represents the norm.

Thus the thymus glands of castrated or adrenalectomized animals on the Rockland diet, although they are larger than the thymus glands of seemingly comparable animals on the same diet, cannot be considered as enlarged or hyperplastic. Such castrated or adrenalectomized animals rather fail to show evidence of thymic atrophy, when they are exposed to the same stress of suboptimal conditions as their controls.

## SUMMARY

The thymus weight in relation to body weight of castrated or adrenalectomized male albino rats of the Sherman strain was compared with that of intact animals.

Differences in the thymus weight were observed, only when castrated or adrenalectomized animals were compared with "control animals" which were below their proper body size. The thymus weight in relation to body weight of optimally growing rats, or of such animals which recovered fast enough from previous growth restraint was not different from that of castrated or adrenalectomized animals.

## CONCLUSION

In the male albino rat the normal course of thymic development is not changed after castration or adrenalectomy. In castrated or adrenalectomized animals under suboptimal conditions the thymus shows less underdevelopment or atrophy than does the thymus of non-operated animals under similar conditions.

I wish to thank Dr. T. F. Zucker for his stimulating interest and criticism and Mrs. Claudia French for her technical assistance.

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# THE INTRASPLENIC INJECTION OF SOME SYNTHETIC ESTROGENS AND PROESTROGENS

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WE HAVE previously reported that the intrasplenic injection of natural estrogens permits the evaluation of the role of the liver in their inactivation in vivo (Segaloff, 1943).

The present study was undertaken in an attempt to ascertain if similar evidence could be obtained regarding the metabolism of synthetic estrogens and proestrogens.

## MATERIALS AND METHODS

Intrasplenic injections were made exactly as in our previous studies. (Segaloff, 1943.) The required amount of estrogen was injected in 0.05 cc. of 5% benzyl alcohol in sesame oil into spayed female rats which had been primed fourteen days previously with 50 μg. of estrone in 0.1 cc. peanut oil. As suggested by Nicholas (1933) we substituted staples for the skin clips which had been used previously to close skin incisions.

## RESULTS

The results are recorded in Table I. Each figure represents the injection of not less than ten nor more than twenty animals. The three median figures for vaginal estrus, i.e., the 50% level and the levels immediately above and below that figure, for each route of administration for each of the estrogens are presented for purposes of comparison. The ratio of effectiveness for each estrogen was calculated from the amount required for 50% response by assigning unity to the 50% estrus value for injection into the transplanted spleen.

The natural estrogen equilenin and the modified natural estrogen ethinyl estradiol are included to extend the data presented in our earlier paper.

The results with diethylstilbestrol were such that a ratio of 0.2:1:17 was obtained. Although five times as much diethylstilbestrol dipropionate as free diethylstilbestrol was required by subcutaneous

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TABLE I. RESULTS OF ESTROGEN INJECTION BY VARIOUS ROUTES

| | Subcutaneous Injection | | Intrasplenic Injection | | | | Ratio |
|---|---|---|---|---|---|---|---|
| | | | Subcutaneous spleen | | Spleen, in situ | | |
| | Dose | Estrus | Dose | Estrus | Dose | Estrus | SC:ISSC:IS[1] |
| | μg. | % | μg. | % | μg. | % | |
| Diethylstilbestrol | 0.25 | 10 | 1.00 | 30 | 10.00 | 0 | |
| | 0.33 | 50 | 1.50 | 50 | 25.00 | 50 | 0.2:1:17 |
| | 0.50 | 80 | 2.00 | 80 | 40.00 | 70 | |
| Diethylstilbestrol dipropionate | 1.00 | 30 | 0.25 | 20 | 1.00 | 20 | |
| | 1.50 | 50 | 0.50 | 50 | 1.75 | 50 | 3:1:3.5 |
| | 2.00 | 80 | 1.00 | 100 | 2.50 | 80 | |
| Diethylstilbestrol dimethyl ether | 10.00 | 30 | 7.50 | 20 | 7.50 | 10 | |
| | 15.00 | 50 | 15.00 | 50 | 15.00 | 50 | 1:1:1 |
| | 20.00 | 100 | 20.00 | 80 | 20.00 | 100 | |
| Hexestrol | 0.25 | 10 | 0.50 | 30 | 20.00 | 20 | |
| | 0.33 | 50 | 1.00 | 50 | 22.50 | 50 | 0.3:1:22.5 |
| | 0.50 | 80 | 1.50 | 70 | 25.00 | 80 | |
| 2,4-di(parahydroxy phenyl)3-ethyl hexane (118B, octofollin) | 0.50 | 0 | 0.75 | 30 | 7.50 | 20 | |
| | 0.75 | 50 | 1.00 | 50 | 10.00 | 50 | 0.75:1:10 |
| | 1.00 | 70 | 1.50 | 80 | 15.00 | 90 | |
| 2,4-di(parahydroxy phenyl)-3-ethyl hexane diacetate | 2.00 | 20 | 3.00 | 20 | 20.00 | 30 | |
| | 3.00 | 50 | 4.00 | 50 | 35.00 | 50 | 0.75:1:9 |
| | 4.00 | 80 | 6.00 | 80 | 50.00 | 80 | |
| Triphenylethylene | 1000.00 | 20 | 1000.00 | 30 | 500.00 | 20 | |
| | 2000.00 | 50 | 2000.00 | 50 | 600.00 | 50 | 1:1:0.3 |
| | 3000.00 | 90 | 3000.00 | 80 | 750.00 | 80 | |
| Triphenylchlorethylene | 10.00 | 0 | 20.00 | 20 | 5.00 | 30 | |
| | 20.00 | 50 | 40.00 | 50 | 6.00 | 50 | 0.5:1:0.15 |
| | 40.00 | 90 | 60.00 | 80 | 7.50 | 80 | |
| 9,10-di-n-propyl-9,10-dehydro-9,10-dihydroxy-1:2:5:6 dibenzanthracene | 10.00 | 10 | 10.00 | 20 | 2.50 | 20 | |
| | 25.00 | 50 | 25.00 | 50 | 5.00 | 50 | 1:1:0.2 |
| | 50.00 | 80 | 50.00 | 80 | 10.00 | 80 | |
| Equilenin | 100.00[2] | 20 | 100.00[2] | 0 | 100.00[2] | 0 | |
| Ethinyl estradiol | 0.10 | 20 | 0.10 | 20 | 5.00 | 30 | |
| | 0.18 | 50 | 0.18 | 50 | 7.50 | 50 | 1:1:42 |
| | 0.25 | 80 | 0.25 | 70 | 10.00 | 80 | |

[1] SC, subcutaneous injection; ISSC, intrasplenic injection into spleen transplanted to subcutaneous position and without portal drainage; IS, injection into spleen in situ.
[2] Maximum solubility.

injection, sufficient protection from inactivation by the liver was afforded by the esterification to yield a ratio of 3:1:3.5. Similarly methylation to diethylstilbestrol dimethyl ether raised the subcutaneous threshold (ten times that observed for diethylstilbestrol dipropionate), affording even greater protection from destruction by the liver and giving a ratio of 1:1:1.

Hexestrol acted in a manner similar to diethylstilbestrol, the ratio of activity being 0.3:1:22.5.

2,4-di(parahydroxy phenyl)3-ethyl hexane was only a little less than one half as active as diethylstilbestrol, but it yielded the more favorable ratio of 0.75:1:10. Unlike diethylstilbestrol dipropionate 2,4-di(parahydroxy phenyl)3-ethyl hexane diacetate has almost the same ratio as the free compound, namely 0.75:1:9.

In direct contrast to the compounds listed above, triphenylethylene, with a ratio of 1:1:0.3, was considerably more active when it was caused to pass through the liver before entering the systemic circulation. The addition of an atom of chlorine to produce triphenylchlorethylene makes a profound difference in the subcutaneous threshold, the latter being one one-hundredth of that for triphenylethylene. However, it retains a ratio indicating even greater activity when injected into the spleen *in situ*, 0.5:1:0.15. 9,10-di-n-propyl-9,10-dehydro-9,10-dihydroxy-1:2:5:6 dibenzanthracene, although not structurally related to the latter two compounds, nevertheless displayed the same type of ratio, namely 1:1:0.2.

The solubility of equilenin is such that only 100 μg. is soluble in the 0.05 cc. of the solvent which was employed routinely in these experiments. Twenty per cent of the animals given 100 μg. by subcutaneous injection came into estrus and none of the animals given 100 μg. by intrasplenic injection displayed estrous vaginal smears.

Although ethinyl estradiol was the most effective by subcutaneous injection of the compounds which we have studied, its ratio is 1:1:42.

## DISCUSSION

It is apparent that diethylstilbestrol is inactivated by the liver since seventeen times as much is required for the production of vaginal estrus in 50% of the animals when the spleen is *in situ* with the portal drainage intact as when injection is made into a transplanted spleen divorced from the hepatic portal system. Hexestrol has essentially the same activity as diethylstilbestrol by all routes of administration. This seems to indicate that the two substances have similar modes of inactivation.

Although diethylstilbestrol has gained widespread use both in experimental and clinical work there are very few references relative to the role of the liver in its inactivation. Allen (1941) reported that pellets of diethylstilbestrol placed in the portal field produced vaginal estrus in spayed female rats. Since pellets of estrone similarly placed failed to produce estrus (Biskind and Mark, 1939) this was interpreted by Allen as indicating that, unlike estrone, diethylstilbestrol is not inactivated in the liver. The most likely explanation of this phenomenon is that the absorption from the diethylstilbestrol pellets was greater than from the estrone pellets, since when a sufficient number of estrone pellets are similarly implanted vaginal estrus results (un-

published data). Zondek, Sulman and Sklow (1943) have recently reported that the liver is capable of inactivating diethylstilbestrol in vitro, but that less diethylstilbestrol than estrone is inactivated by the same amount of liver.

The results with diethylstilbestrol dipropionate increase the likelihood that the metabolic destruction or inactivation by the liver of natural estrogens and of diethylstilbestrol is similar. The ratio for diethylstilbestrol dipropionate is one-quarter of that for the free compound. This would indicate that the inactivation proceeds at least partially through the hydroxyl group. This argument is further strengthened by the fact that diethylstilbestrol dimethyl ether, which has a stronger bond chemically than an ester, has the same degree of activity by all routes of administration.

The greater activity of diethylstilbestrol dipropionate by injection into the subcutaneously transplanted spleen than by subcutaneous injection brings to mind the similar situation which exists in the case of certain esters of $\alpha$-estradiol. Again we must consider two possibilities, namely that the spleen is capable of either hydrolyzing the ester, or that it is absorbed at a more effective rate from the vascular spleen. Heller's (1940) *in vitro* work would favor the first interpretation.

2,4-di(parahydroxy phenyl)3-ethyl hexane is inactivated to a lesser degree than is diethylstilbestrol although its peripheral activity, as indicated by the threshold for subcutaneous injection, is only about one-half that of diethylstilbestrol. The ratio for its diacetate is the same as the ratio for the free compound. This can be interpreted as meaning either that the acetate is very readily hydrolyzed, or that inactivation of this compound does not proceed through the hydroxyl groups.

In direct contrast to the compounds already mentioned are triphenylethylene, triphenylchlorethylene and 9,10-di-n-propyl-9,10-dehydro-9,10-dihydroxy-1:2:5:6 dibenzanthracene. These three compounds have their peripheral activity greatly enhanced by passage through the hepatic portal system. It is of extreme interest that these compounds are classified as proestrogens by Emmens (1941) who states that proestrogens do not act directly on the end organ, but are changed elsewhere in the body into substances which can act on the end organs. It would appear likely from the results which are reported here that the transformation from proestrogen to estrogen, suggested by Emmens, takes place in the liver.

Since these compounds which undergo potentiation are phenyl compounds and since conversion to phenols, at least in the case of triphenylethylene, would increase the estrogenic potency (Emmens, 1943) it seems reasonable to assume that the liver converts part of the compound into phenolic homologues. The possibility that this latter transformation may occur is strengthened by the isolation of

phenolic estrogens from urine after the injection of phenyl proestrogens (Stroud, 1940).

Contrary to expectations, ethinyl estradiol showed a ratio very similar to that of $\alpha$-estradiol or of $\alpha$-estradiol-3-benzoate. It is, however, by far the most active derivative of estradiol which we have tested by subcutaneous injection (Segaloff, 1943). Thus, it would appear likely that the high oral potency of ethinyl estradiol is due to its high peripheral activity rather than to a failure of inactivation by the liver.

## SUMMARY

Diethylstilbestrol and hexestrol are inactivated in the liver, but to a lesser extent than estrone or $\alpha$-estradiol. The inactivation is related to the hydroxyl groups since esterification or methylation reduces the degree of inactivation.

Ethinyl estradiol is the most active derivative of $\alpha$-estradiol, but it is inactivated in the same proportion as is free estradiol. This suggests that the marked oral activity of ethinyl estradiol is due to its high potency rather than its resistance to hepatic inactivation.

Triphenylethylene, triphenylchlorethylene and 9,10-di-n-propyl-9,10-dehydro-9,10-dihydroxy-1:2:5:6 dibenzanthracene are all potentiated in estrogenic activity by prior passage through the liver.

It is suggested that passage through the liver is an important factor in the conversion of proestrogens to estrogens.

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# THE INFLUENCE OF THE SEX HORMONES ON THE BURSA OF FABRICIUS AND THE PELVIS IN THE RING-NECKED PHEASANT[1]

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IT HAS LONG been known that the bursa of Fabricius, a dorsal diverticulum of the cloaca, is characteristically present in young birds but is almost always absent in the adult with the exception of the Ratitae. Jolly (1915) referred to the bursa as a "cloacal thymus," reported that in the fowl maximum bursa size occurred at approximately four months, and that initiation of bursa involution coincided with the appearance of sexual maturity. Riddle (1928) found that in doves and pigeons the ovaries and testes developed slowly and appeared to be suppressed during the period of rapid body, bursa, and thymus growth. Involution of the bursa began immediately following its maximum development, and was usually complete at the time of sexual maturity. The period of involution coincided with the phase of rapid gonad devlopment and the relationship of the bursa to the growth of the gonads was regarded as suggestive evidence of an endocrine function of the bursa.

The function of the bursa is not clear. Riddle and Tange (1928) found that the removal of the bursa had no apparent effects in ring doves. Later work by Riddle and Krizenecky (1931) indicated that removal of both thymus and bursa produced no noticeable changes in pigeons.

Recently the bursa has been studied as an age indicator in game birds. From observations on live pheasants, Linduska (1943) and Kirkpatrick (1944a) found the bursa to be indicative of sexual immaturity from hatching (May) until the following spring. The bursa is usually completely involuted in pheasants which have passed through a breeding season (April to July). In a series of 177 pheasants ranging from 16 to 362 days of age, Kirkpatrick (1944b) found that maximum growth of the thymus and bursa was attained during the period of

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[2] We are indebted to Dr. Erwin Schwenk of the Schering Corporation, Bloomfield, New Jersey, for the testosterone propionate used in this study and to Merck and Company, Rahway, New Jersey, for generous supplies of diethylstilbesterol.

most rapid body growth and during the time when the gonads were inactive. The bursa and thymus reached their maxima at 130 days of age (September) and began simultaneous involution immediately. The initiation of involution of these organs was correlated with a marked increase in testis size (Kirkpatrick and Andrews, 1944). A large number of bursa measurements from live pheasants by Kirkpatrick (1944a) and Linduska revealed bursa involution as a continuous process from September and October respectively, with the complete atrophy of the organ occurring just prior or during the breeding season.

Kirkpatrick (1943) found that in sexually immature female pheasants the injection of pregnant mare's serum greatly reduced bursa depth and tripled ovarian weight. Kirkpatrick and Andrews (1944) found that simultaneously with the onset of bursa involution in pheasants, the testes showed intense spermatogenic activity with the production of spermatozoa in some males, although normally the pheasant is restricted to a definite spring breeding period. These observations suggested a relationship between bursa and thymus involution and gonad activity. The object of the present investigation was to determine the influence of the male and female sex hormones upon bursa involution.

## MATERIALS AND METHODS

Seventy-six pheasants, 15 weeks of age were divided into the following groups: *Group I.* 12 normal male controls. *Group II.* 17 normal males injected with 0.3 mg. of testosterone propionate daily for 20 days. *Group III.* 19 males castrated at 14 weeks of age. *Group IV.* 18 males castrated at 14 weeks of age and injected with 0.3 mg. of testosterone propionate daily for 20 days beginning at the 15th week of age. *Group V.* 20 normal females injected with 0.2 mg. of stilbesterol daily for 20 days. Thirty-eight normal females of the same age were observed as a check group at the Jasper-Pulaski State Game Farm.

Injections were made subcutaneously in the pectoral region. Bursa depth was measured by the technique described by Kirkpatrick (1944a). Measurements were made prior to hormone injection, at weekly intervals for 8 weeks, and at 5 week intervals thereafter. The distance between the pubic bones of the injected females was measured on the day following the last injection, and similar measurements were made on 38 untreated females at the game farm.

The pheasants were kept in a large outdoor pen. The normal and castrated males receiving testosterone propionate were placed in one pen, the uninjected normal and castrated males were kept in a separate compartment, and the stilbesterol injected females were isolated in a third pen.

## RESULTS

A summary of the changes in bursa depth is shown in Figure 1. In the untreated normal and castrated males, bursa involution proceeded slowly and at a nearly constant rate from September 27 until Febru-

ary 3. The curves for both the intact and castrated males were closely similar and are representative of the type and rate of involution which occurs in normal males and females at this season of the year (Linduska, 1943; Kirkpatrick, 1944a). In normal and castrated males treated with 0.3 mg. of testosterone propionate daily for 20 days and in normal females injected with 0.2 mg. of diethylstilbesterol daily

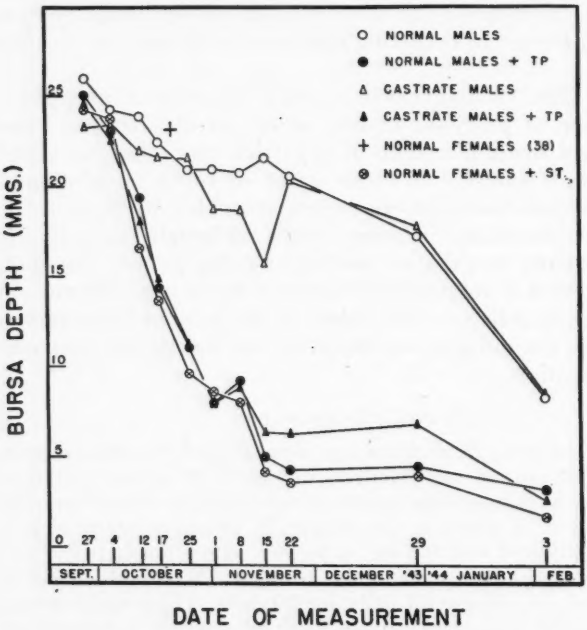


FIG. 1. The influence of testosterone propionate and diethylstilbesterol upon the rate of involution of the bursa in the ring-necked pheasant.

for the same length of time, the rate of involution was markedly increased. Decreases in bursa depth were observed as early as the 14th day of injection and involution proceeded rapidly until the average bursa depth of the injected groups was approximately 5 mm. on November 15. During the 20 day period of hormone injection the average decrease in the depth of the bursa was approximately 10 mm. in the testosterone- and stilbesterol-treated pheasants and only 2 mm. in the controls. Measurements of bursa depth in 38 normal females (of the same hatch) at the State Game Farm showed that the degree of bursa involution in these females was similar to that in the normal and castrated control males at the Purdue Station, indicating that the events observed in the hormone injected birds represent physiological changes not explained by variations in the normal pheasant popula-

tion. In the hormone-treated birds bursa depth was but slightly reduced after November 15 and the average depth was less than 3 mm. when the experiment was terminated on February 3. In the untreated normal and castrated male controls bursa involution progressed at a nearly constant rate and depth had been reduced to approximately 8 mm. when the study was ended.

Although the present experiment was designed to measure the effects of sex hormones on the bursa, several other general hormonal changes were observed. In the stilbesterol-injected females the vent had become dilated, moistened and pliable at the end of the first week of injections and the pubic bones had increased in elasticity and had

TABLE 1. EFFECT OF STILBESTEROL ON THE DISTANCE BETWEEN THE PUBIC BONES IN THE FEMALE PHEASANT

| Treatment | No. of Measurements | Average Distance Between Bones |
|---|---|---|
| | | *mm.* |
| Controls | 38 | 13.1 |
| Stilbesterol | 16 | 24.0 |

begun to move apart. These cloacal changes became more pronounced as the hormone treatment continued and at the end of the 20 day injection period the entire cloaca was enlarged, moist, highly vascular and the oviduct could be extruded by slight pressure upon the abdomen. As shown in Table 1, the distance between the pubic bones was doubled in the injected birds. The physiological changes induced by stilbesterol were closely similar if not identical to those which are observed in birds during the laying season. In the testosterone-injected normal and castrated control males approximately 50 per cent showed grossly hypertrophied copulatory organs and in others the wattles were apparently enlarged. These changes were not observed in any of the untreated normal or castrated control males. Several of the injected males attempted to tread the hens when the two sexes were allowed to run together in their pens but neither crowing nor "tidbitting" (normal male sexual behavior) were observed.

## DISCUSSION

The reports of Jolly and Pezard (1928), as cited by Riddle and Krizenecky (1931), that castration delays bursa involution in the domestic fowl, of Kirkpatrick (1943) that the injection of pregnant mare's serum gonadotropin hastens bursa involution in sexually immature female pheasants, and of Kirkpatrick and Andrews (1944) that in normal male pheasants the onset of bursa involution is coincident with a brief period of intense spermatogenesis in late summer and early fall, strongly suggest that the development and function of the

gonads is in some way related to bursa involution. The results obtained in the present investigation provide additional evidence of an inter-relationship between these organs and indicate that the rate of involution of the bursa can be greatly increased by the injection of either testosterone propionate or diethylstilbesterol.

The castration of male pheasants at 14 weeks of age had no apparent effect upon the rate of involution of the bursa. This finding is in contrast with the report of Jolly and Pezard on the domestic fowl and does not fit a tentative hypothesis that if the injection of the sex hormones increases the rate of involution the removal of the natural sources of the hormones should delay regression of the bursa. Although other experiments must be carried out before the influence of castration upon the bursa can be known, several suggestions for the failure of castration to delay involution present themselves.

The fact that bursa involution in the normal male pheasant is preceded by a brief period of increased testicular activity at 12–14 weeks of age may set up certain physiological processes which can proceed to completion in the absence of the testis after this period. It is possible that had castration been performed prior to this period of increased spermatogenesis, which may also be accompanied by increased androgen production, the results might have been different. It will be necessary, therefore, to investigate the age at the time of castration in relation to the bursa. A second explanation for the failure of gonadectomy to affect the bursa is the possibility that extratesticular sources of androgens may be sufficient to initiate bursa involution. Although the evidence gathered in the present study clearly indicates that androgens and estrogens are capable of hastening bursa involution, it is possible that other substances may likewise affect the involution of the bursa of Fabricius.

## SUMMARY

The influence of testosterone propionate and diethylstilbesterol on the bursa of Fabricius has been studied in 56 male and 20 female ring-necked pheasants. In normal and castrated males injected with 0.3 mg. of testosterone daily for 20 days beginning at the 15th week of age the rate of involution of the bursa was increased in comparison with uninjected normal and castrated controls. The injection of normal females of similar age with 0.2 mg. of stilbesterol daily for 20 days induced the same rate of involution as did testosterone. The castration of male pheasants at 14 weeks of age did not affect the bursa, and involution progressed at the usual seasonal rate which is observed in intact males and females. It is concluded that the rate of involution of the bursa can be increased by the injection of either testosterone propionate or diethylstilbesterol.

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# THE ROLE OF THE VITAMINES OF THE B-COMPLEX IN ESTROGEN METABOLISM

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BISKIND AND Biskind (1942) have demonstrated that vaginal cornification does not occur in castrated female rats, fed a normal diet, in which estrone pellets have been implanted intrasplenically. This finding was interpreted as being due to the inactivation of estrone by the liver. However, when the animals were subsequently fed a B-complex deficient diet, most of them displayed vaginal estrus. Supplementation of the diet with yeast returned the animals to the anestrous state.

We have previously reported (Segaloff, 1943) the inactivation by the rat liver of estrone and $\alpha$-estradiol intrasplenically injected. In the near future a similar report will be made for diethylstilbestrol.

## MATERIAL AND METHODS

The rats used in this study were all females of the Sprague-Dawley strain purchased from a local dealer. The basal colony diet was "Rockland Farms" rat chow upon which all animals had been maintained before being placed on the experimental diets. All rats were spayed prior to the shift to experimental diets.

Two experimental diets were employed (table 1). Diet #1 was fed in the case of the animals used as calorie controls and at a level which was based upon the average consumption of 15 animals on Diet #2, the B-complex deficient diet. Diet #1 was prepared in such a way that the daily allowance for each animal contained 0.5 gm. of Anheuser-Busch's Strain G brewer's yeast. The animals were fed a total of 9.0 gms. per day for the first two weeks, 6.5 gms. per day for the third week, 4.0 gms. per day for the fourth week, and 2.0 gms. per day for the fifth week.

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TABLE 1

| | Diet 1 | Diet 2 |
|---|---|---|
| Casein (non-extracted) | 20 | |
| Casein (vitamin-free)* | | 20 |
| Cornstarch | 64 | |
| Sucrose | | 64 |
| Crisco | 10 | 10 |
| Salts (U.S.P. XI) | 4 | 4 |
| Cod liver oil | 2 | 2 |

* S.M.A. Co., Chagrin Falls, Ohio.

Various supplements were added to the B-complex deficient diet. Table 2 shows the supplements and the levels employed. The first level was fed for three weeks, the second level for the fourth week, and the third level for the fifth week. Each supplement was fed individually.

TABLE 2

| | 1st level (mg./100 grams) | 2nd level (mg./100 grams) | 3rd level (mg./100 grams) |
|---|---|---|---|
| Choline chloride | 400.0 | 800.0 | 1600.0 |
| Pyridoxin | 1.2 | 2.4 | 4.8 |
| Riboflavin | 1.6 | 3.2 | 6.4 |
| Thiamin hydrochloride | 0.8 | 1.6 | 3.2 |
| Calcium pantothenate | 1.0 | 2.0 | 4.0 |

The method of intrasplenic injection described in our previous studies (Segaloff, 1943) was used. $\alpha$-Estradiol, estrone or diethylstilbestrol dissolved in 0.05 cc. of 5% benzyl alcohol in sesame oil was injected into the spleen or injected subcutaneously. The assay injections were made after 35 days on the experimental diet. Fourteen days previously the rats had been primed with 50 micrograms of estrone in 0.1 cc. peanut oil.

## RESULTS

Table 3 shows the effect of the diets on the weight of the experimental animals. It should be noted that all rats on deficient Diet #2, except those which received thiamin chloride, lost from 33 to 44 grams in 5 weeks. The calorie controls on Diet #1 lost 25 grams.

When rats were fed the B-complex deficient diet the vaginal response to estrogen injected intrasplenically was increased over that of the normally fed control animals. This is interpreted as meaning that the B-complex deficiency affected adversely the enzyme systems of the liver which are concerned with the inactivation of estrogens and that this was accomplished in the absence of histologically evident hepatic damage. The data also suggested that in rats which had been fed a B-complex deficient diet the vaginal response to estrogen injected subcutaneously was decreased as compared to that of normally fed control rats. This is interpreted as due to a reduction in end organ response to estrogenic hormone. This decreased response of end organs to circulating estrogen would increase the significance of the greater response to estrogen injected intrasplenically.

It is important to note that while the reaction of the calorie controls to estrone and $\alpha$-estradiol is approximately the same as in the controls fed ad libitum, the calorie controls injected with diethylstilbestrol showed a response which was intermediate between that of the normal controls and of the B-complex deficient rats.

The results with the various supplements were basically the same for estrone and $\alpha$-estradiol. Supplementing the B-complex deficient diet (Diet #2) with choline chloride, pyridoxin or calcium pantothenate failed to increase the rate of inactivation of estrogen. The use

TABLE 3

| Diet | Supplement: | Weight of rats in grams: | | |
|---|---|---|---|---|
| | | Initial | At assay | Loss |
| I. Calorie control: | | | | |
| | Yeast | 137 | 112 | 25 |
| II. B-complex deficient: | | | | |
| | None | 136 | 92 | 44 |
| | Choline Chloride | 125 | 85 | 40 |
| | Pyridoxin | 132 | 98 | 34 |
| | Riboflavin | 127 | 94 | 33 |
| | Thiamin Hydrochloride | 130 | 126 | 4 |
| | Calcium Pantothenate | 133 | 99 | 34 |
| | Calcium Pantothenate, Choline, Pyridoxin | 123 | 93 | 30 |
| | Thiamin Hydrochloride, Calories Restricted | 124 | 115 | 9 |

of either riboflavin or thiamin hydrochloride, on the other hand, did increase the rate of inactivation to a level which was essentially normal.

The results with diethylstilbestrol in the animals on the B-complex deficient diet (Diet #2) and on the calorie control diet were in striking contrast to the findings in the case of estrone and $\alpha$-estradiol. None of the supplements which were employed returned the rate of inactivation of diethylstilbestrol to a normal level.

In order to eliminate the effect of thiamin on appetite as a possible factor in its effect on estrogen metabolism, a group of animals was fed Diet #2 supplemented with thiamin, but restricted to an amount equal to that consumed by the animals on Diet #2 without supplementation. The animals were then injected with $\alpha$-estradiol either subcutaneously or intrasplenically. They lost a moderate amount of weight during the experimental period, but the results, as seen in table 4, demonstrate that the effect of thiamin is not due to its effect on appetite.

An additional series of animals was fed Diet #2 supplemented with choline chloride, pyridoxin and calcium pantothenate with the results seen in table 4. It is apparent that the results in these animals do not differ from those seen in the group given basal ration only or in the

TABLE 4*

| Diet | Supplement | Route of estrogen injection | α-Estradiol | | Estrone | | Diethylstilbestrol | |
|---|---|---|---|---|---|---|---|---|
| | | | Dose micrograms | Estrus per cent | Dose micrograms | Estrus per cent | Dose micrograms | Estrus per cent |
| Normal[1] | None | Intrasplenic | 50<br>100 | 20<br>70 | 140 | 20 | 10<br>20 | 0<br>20 |
| | | Subcutaneous | 1<br>2 | 30<br>70 | 4<br>8 | 40<br>60 | 0.25<br>0.5 | 0<br>80 |
| 1[2] | Yeast | Intrasplenic | 50<br>100 | 0<br>80 | 140 | 0 | 5<br>10 | 20<br>40 |
| | | Subcutaneous | 1<br>2 | 0<br>60 | 8<br>16 | 40<br>80 | 0.25<br>0.5 | 40<br>80 |
| 2[3] | None | Intrasplenic | 25<br>50 | 20<br>60 | 75<br>140 | 40<br>60 | 5<br>10 | 0<br>80 |
| | | Subcutaneous | 2<br>4 | 0<br>60 | 8<br>16 | 40<br>60 | 0.5<br>1.0 | 40<br>80 |
| | Choline Chloride | Intrasplenic | 25<br>50 | 0<br>60 | 75<br>140 | 40<br>100 | 5<br>10 | 60<br>60 |
| | | Subcutaneous | 2<br>4 | 0<br>40 | 8<br>16 | 40<br>80 | 0.25<br>0.5 | 40<br>60 |
| | Pyridoxin | Intrasplenic | 25<br>50 | 20<br>80 | 75<br>140 | 0<br>60 | 5<br>10 | 60<br>80 |
| | | Subcutaneous | 1<br>2 | 20<br>60 | 4<br>8 | 40<br>60 | 0.5<br>1.0 | 40<br>80 |
| | Riboflavin | Intrasplenic | 50<br>100 | 20<br>60 | 140 | 20 | 5<br>10 | 20<br>60 |
| | | Subcutaneous | 2<br>4 | 40<br>20 | 8<br>16 | 40<br>60 | 0.25<br>0.5 | 30<br>60 |
| | Thiamin Hydrochloride | Intrasplenic | 50<br>100 | 0<br>60 | 140 | 20 | 10<br>20 | 40<br>40 |
| | | Subcutaneous | 2<br>4 | 0<br>60 | 4<br>8 | 20<br>80 | 0.25<br>0.5 | 20<br>60 |
| | Calcium Pantothenate | Intrasplenic | 25<br>50 | 60<br>60 | 75<br>140 | 60<br>80 | 5<br>10 | 40<br>60 |
| | | Subcutaneous | 1<br>2 | 50<br>80 | 4<br>8 | 60<br>60 | 0.5<br>1.0 | 20<br>100 |
| Choline Chloride, Pyridoxin, Calcium Pantothenate | | Intrasplenic | 25<br>50 | 60<br>80 | 75<br>140 | 20<br>100 | 5<br>10 | 60<br>80 |
| | | Subcutaneous | 1<br>2 | 0<br>60 | 8<br>16 | 20<br>80 | 0.25<br>0.5 | 20<br>80 |
| Thiaminhydrochloride, Restricted Calories | | Intrasplenic | 50<br>100 | 0<br>40 | | | | |
| | | Subcutaneous | 2<br>4 | 20<br>60 | | | | |

* In this table each figure for the normal animals represents from 10 to 20 animals. All other figures represent 5 animals.
[1] Rockland farms rat pellets. [2] Calorie control. [3] B-complex deficient.

groups fed these same supplements individually. Since these figures agree so closely they may be regarded as additional evidence that none of these members of the B-complex are responsible for the relation of the latter to the metabolism of estrogen.

## CONCLUSIONS

Deficiency in the vitamines of the B-complex decreases the ability of the rat's liver to inactivate estrone and α-estradiol. Such a deficiency also decreases the response of the rat to α-estradiol injected subcu-

taneously. In these rats, the response to other estrogens injected subcutaneously showed the same general tendency. The addition of choline chloride, pyridoxin or calcium pantothenate to the deficient diet fails to affect the deficient hepatic inactivation of estrone and $\alpha$-estradiol. In contrast to this, thiamin hydrochloride and riboflavin are capable of restoring the ability of the liver to inactivate estrone and $\alpha$-estradiol to normal levels. The effect of thiamin hydrochloride is independent of its effect on appetite. Both inanition and B-complex deficiency greatly decrease the ability of the rat's liver to inactivate diethylstilbestrol. Choline chloride, pyridoxin, riboflavin, thiamin hydrochloride, and calcium pantothenate all failed to restore this deficient inactivation to normal.

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# NOTES AND COMMENTS

## METABOLISM OF THE STEROID HORMONES: THE CONVERSION OF TESTOSTERONE TO ANDROSTERONE BY THE PREGNANT RHESUS MONKEY[1,2]

ANDROSTERONE is one of the metabolites formed from testosterone and excreted in the urine in man (Callow, 1939; Dorfman, Cook and Hamilton, 1939) and in male chimpanzees (Fish and Dorfman, 1944). We now have evidence that this conversion also takes place in the pregnant monkey.

*Monkey Pregnancy After Administration of Testosterone Propionate*

Testosterone in the form of its propionate was administered to three pregnant Rhesus monkeys. The hormone was injected subcutaneously in corn oil which contained 25 mg. testosterone propionate per cc.

*Monkey A.* The urine was collected from the 45th day of pregnancy to the 67th, during which time the animal received sixteen injections equivalent to 400 mg. of testosterone propionate.

*Monkey B.* The urine was collected from the 49th day of pregnancy to the 69th, during which time the animal received thirteen injections equivalent to 325 mg. of testosterone propionate.

*Monkey C.* The urine was collected from the 37th day of pregnancy to the 66th, during which time the animal received nineteen injections equivalent to 475 mg. of testosterone propionate.

The total urine collection represented 70 days and amounted to 13.7 liters. A total of 1.2 gm. of testosterone propionate[3] was administered.

The urine was acidified with hydrochloric acid and extracted with benzene as previously described (Fish and Dorfman, 1942). The neutral fraction, which weighed 5.2 gm., was separated with the Girard-Sandulesco reagent, trimethylacethydrazide ammonium chloride (6 gm. reagent, 6 cc. glacial acetic acid, 54 cc. absolute alcohol) and a ketonic fraction weighing 1.1 gm. was obtained. This was dissolved in 25 cc. hot 90 per cent ethanol and 25 cc. of a hot 2 per cent digitonin solution in 90 per cent ethanol were added. The digitonide was centrifuged and washed with 90 per cent ethanol and ether.

The mother liquor and washings were combined and evaporated *in vacuo*. The dried residue was dissolved in pyridine and warmed on a steam bath. An

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[2] Aided by a grant from the Rockefeller Foundation and by a grant from the Fluid Research Fund of the Yale University School of Medicine.

[3] Testosterone propionate was furnished by Ciba Pharmaceutical Products, Inc., under the trade name Perandren.

excess of ether was added, the pyridine removed, and on evaporation of the ether, an oily residue representing the ketonic-, digitonin-soluble portion was obtained.

This residue was dissolved in 25 cc. carbon tetrachloride and adsorbed on a column of Brockmann's aluminum oxide. A yellow oil was obtained from the eluate from 150 cc. carbon tetrachloride. The column was then eluted with 50 cc. of 0.1 per cent ethanol in carbon tetrachloride. This eluate yielded a crystalline residue. After recrystallization from methanol, the compound melted at 175–178° C.[4] The yield of recrystallized product was 15 mg. An acetate was made with a small portion of the compound. After four recrystallizations from aqueous methanol, the acetate melted at 160–161° C. When mixed with an authentic sample of androsterone acetate (m.p. 161° C.), the mixture melted at 160–161° C.

Efforts to recover more androsterone from the mother liquors yielded only oils. Therefore, an attempt was made to purify this oil by way of the acetate. After two recrystallizations from aqueous methanol, 8.5 mg. of the acetate (7.4 mg. of androsterone) were obtained, which melted at 159–160° C. and gave no depression when mixed with an authentic sample of androsterone acetate. Thus a total of 22 mg. of androsterone was isolated, representing a 2.5 per cent conversion of testosterone to androsterone.

An attempt to isolate androsterone from the urine of pregnant monkeys not receiving testosterone propionate was unsuccessful even though 2.6 times as much urine was used. A total of 49.4 liters of urine representing 182 days of urine was collected and studied in a manner similar to that used for the urine collected after testosterone propionate administration. Marker and Hartman (1940) were unable to isolate androsterone from the urine of pregnant monkeys.

SUMMARY

A total of 1.2 grams of testosterone propionate was administered to three pregnant Rhesus monkeys and the urine collected for seventy days. From this urine a total of 22 mg. of androsterone was isolated. An attempt to isolate androsterone from the urine of pregnant monkeys not receiving testosterone was unsuccessful even though 2.6 times as much urine was used.

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[4] All melting points are uncorrected.